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The Stability of Tea Polyphenols as a Food Additive and Its Effect on Streptococcus mutans

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ABSTRACT

Dental caries is one of the most concerning chronic diseases, *Streptococcus mutans* is one the most prominent contributing bacterium. *S. mutans* metabolizes sugar in processed foods to demineralize the tooth's surface leading to tooth caries. This study focuses on whether green tea polyphenols epigallocatechin gallate (EGCG), and/or its modifications, palmitoyl-epigallocatechin gallate (P-EGCG) and epigallocatechin-3-gallate-stearate (EGCG-S), can reduce the number of *S. mutans* in different concentrations of sucrose. A colony forming unit assay was utilized to test viability. Results suggested that the tea polyphenols were efficient in inhibiting *S. mutans* up to 5 grams of sucrose per 100ml of artificial saliva ($P < 0.05$). Then the polyphenols were assessed as a potential additive for cavity-causing foods inhibiting the growth of bacteria on the tooth's surface. Using 60% and 70% dark chocolate as a marker, the results indicated three polyphenols are effective in reducing *S. mutans*. EGCG-S and P-EGCG inhibited *S. mutans* significantly in the 60% dark chocolate with an inhibition rate of $70.0\% \pm 1.90\%$ and $81.7\% \pm 2.69\%$, respectively ($P < 0.05$). This study suggests that EGCG, EGCG-S, and P-EGCG have the potential to be used as food additives for dark chocolate.

Keywords: Epigallocatechin gallate (EGCG), dental caries, *Streptococcus mutans*, dark chocolate, tea polyphenols

MONTCLAIR STATE UNIVERSITY

The Stability of Tea Polyphenols as a Food Additive and its Effect on *Streptococcus*
mutan

by

Chia-Liang Huang

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

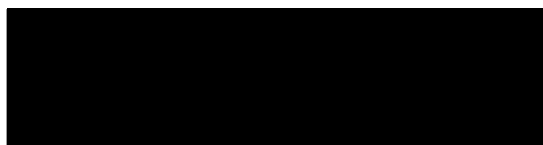
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THE STABILITY OF TEA POLYPHENOLS AS A FOOD ADDITIVE AND ITS
EFFECTS ON *STREPTOCOCCUS MUTANS*

A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science

by

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Montclair, NJ

2018

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1. Introduction

Many types of processed foods are being mass produced due to advances in technology. Food industries usually add large amounts of sugar to make these foods appetizing. This excessive sugar content may lead to serious oral and dental health issues, which may become chronic over time. The most well-known consequence of sugar consumption is dental cavities and the interaction with numerous bacterial species existing in the human mouth. Each bacterium has a unique role in causing different kinds of oral disease; the prominent bacteria that causes tooth decay is *S. mutans* (Loesche, 1986; Valdez et al., 2017). Sugar plays a significant role in the growth of *S. mutans* and researchers have been attempting to discover a substance that can effectively be added to food to inhibit the growth of the bacteria.

Dental caries is one the leading chronic diseases in the world (Gao et al., 2016). According to the World Health Organization, 60-90% of children and nearly 100% of adults have dental caries around the world. There are several causes of dental caries, however, diet undoubtedly plays an important role. Food products that contain sugar are one of the leading reasons for the increasing rate of dental caries in developed countries (Forssten et al., 2010). Sugar is a type of simple carbohydrate, it can be classified as a monosaccharide or disaccharides (Gupta et al., 2013). DMFS stands for decay-missing-filled-surface index; this index describes the amount and prevalence of dental caries in an individual (Rugg-Gunn et al., 1984). This index has been used as a key measure of caries in dental epidemiology for 70 years (Larmas, 2010). The DMFS score of an individual can range from 0 to 148; the higher score of DMFS, the worse the condition is of an individual's teeth (Larmas, 2010). Research indicates that the amount

of sugar intake is strongly related to dental caries (Rugg-Gum et al., 1984). This research showed that 32 children who participated, consumed more than 163 grams of sugar daily and had developed 5 DMFS and another group of 32 children who consumed less than 78 grams of sugar daily developed 3.2 DMFS within the last 2 years. Similarly, another study also agrees that sugar intake is related to having cavities (Burt et al., 1988). Therefore, these studies indicate that sugar intake is correlated with dental caries.

The tooth's surface is covered with a biofilm that contains a large amount of bacterial cells, salivary polymers, and food debris (Forssten et al., 2010). This biofilm can grow thicker without control and form dental plaque. This dental plaque can provide a site for the colonization and growth of many bacterial species, especially *S. mutans* (Forssten et al., 2010). It is a gram-positive and facultative anaerobic cocci bacterium that is commonly discovered in the oral cavity (David et al., 2011). *S. mutans* has been identified as the bacterium that is the primary cause of dental caries and it can utilize sucrose significantly faster than other oral bacteria (Oda et al., 2015; Loesche, 1986; Minah et al., 1977; Onose et al., 1976).

Consuming sucrose and glucose from the food debris that remains on the tooth's surface could help *S. mutans* synthesize extracellular polysaccharides (EPS), namely glucans. These glucans, especially the water insoluble type, contribute the most to the adherence of *S. mutans* and other oral bacterial to the tooth's surface (Xu et al., 2010). This causes the formation of a pathogenic biofilm on the tooth's surface (Yamashita et al., 1993). Glucosyltransferase is an extracellular enzyme that is produced by *S. mutans* (Devulapalle et al., 2001). Glucose also provides rich energy for the synthesis of EPS

(Forssten, 2010). *S. mutans* utilizes sucrose to produce lactic acids. This acid then demineralizes and destroys the tooth enamel, which causes dental cavities (Nassar et al., 2011). Bacterial metabolism of fermentable carbohydrates on the tooth's surface could create acid that damages the enamel and results in a cavity (Nassar et al., 2011). Therefore, foods that contain high amount of carbohydrates (sugar) will have a greater chance of forming dental caries.

Many plaque bacteria synthesize not only EPS, but also intracellular polysaccharides (IPS) from various sugars. When there is lack of exogenous sugar, *S. mutans* could use the stored IPS to start its pathogenicity (Berman et al., 1967; Durso et al., 2014). *S. mutans* is a strong acid producer that causes an acidic environment (Tanzer et al., 2001). Its acidic property can demineralize the tooth's enamel. *S. mutans* is also acid tolerant. During the stage of catabolism of carbohydrates, the pH of dental plaque will become 4.0 or lower, which mean *S. mutans* needs adapts to this acid condition and become acid tolerant (Bender et al., 1986; Griswold et al., 2004). When the plaque acidifies it could cause internal acidification of *S. mutans*. There are several mechanisms that make *S. mutans* acid tolerant, such as a decrease of the proton penetrability of the cell membrane and an increase in the DNA repair pathway; however, membrane-bound $F_1 F_0$ -ATPase is considered to be the key substance of the *S. mutans* that contributes to the acid tolerance (Quivery et al., 2001). Because membrane-bound $F_1 F_0$ -ATPase can push out protons from the cells, it could also maintain the internal pH value and make itself acid tolerant (Bender et al., 1986). Agmatine deiminase system (ADS) is also considered one of the determinants of acid tolerance in *Streptococcus mutans* (Xu et al., 2010). ADS system could enhance the

alkali production which makes *S. mutans* able to persist in an acidic pH environment (Griswold et al., 2004).

Tooth brushing is generally one of the most effective ways to prevent dental caries (Kumar et al., 2016). Several studies indicated that the frequency of brushing is strongly impacted on caries prevention among individuals (McLaren et al., 2016; Tinanoff, N., 2017). However, many factors could affect an individual's brushing habits, such as social position and education level (Rebelo et al., 2015; Fuentes-Garcia et al., 2013; Tsakos et al., 2009,).

A study indicated that the level of education for an individual is related to his/her oral condition, for example, the lower level of education an individual has, the worse oral condition he/she has (Rebelo et al., 2015). The income of an individual may also affect the individual oral condition. Lower-income groups developed higher cavity rate than the higher-income groups (Nomura et al., 2002). Personal oral hygiene is strongly related to the cavity rate, and health education could cause a person to have better personal oral hygiene. However, health education is difficult to apply to several developing countries and to lower-social economic groups. Several studies also indicated that lower-income groups and lower social economic groups have higher dental cavity rates than the average-income groups and higher income groups (Fuentes-García et al., 2013; Kumar et al., 2016).

Recently, the increase in the intake of high-sugar products has resulted in the growth of medical costs for dental caries. Many processed foods contain a high amount of simple carbohydrates. To solve this problem, certain ingredients needed to be added to high-sugar products. One of the most common natural sweeteners that the food

industry has used to replace sucrose is xylitol (Salli et al., 2016). This sweetener is a polyalcohol (sugar alcohol) that is non-cariogenic. Several studies indicated that xylitol could inhibit the growth of *S. mutans* (Yamashita et al., 1993; Soderling et al., 2009). Xylitol is commonly added to chewing gum. However, in addition to sensory issues, the acceptable daily intake of xylitol has not been clearly determined. One study indicates that excessive intake of xylitol may cause diarrhea (Makinen, 2016). Another researcher observed side effects when excessive xylitol was consumed, including cramps, constipation, and oral ulcers (Riley et al., 2015). The other sugar substitute that the food industry has used is maltitol. This sugar substitute is used in many sugar-free products; however, it has been discovered that it can cause a laxative effect and sometimes can cause stomach bloating and gas (Yadav et al., 2014; Chukwuma et al., 2016). Both xylitol and maltitol are sugar alcohols and couldn't be fully broken down by the human digestive system because of the molecular structures of both sugar alcohols. (Grembecka., 2015).

Many investigators have shown that green tea extract, Epigallocatechin gallate (EGCG) is the key component to inhibit the growth of *S. mutans* both *in vitro* and *in vivo* (Hirasawa et al., 2006; Mankovskaia et al., 2013). This could be used as a possible solution to help prohibit dental caries. The literature shows that there are three main antibacterial components of the tea extract that prohibit of the growth of *S. mutans*: gallate (EGCG), galocatechin (GC), and epigallocatechin (EGC). Research also indicates that EGCG inhibits glucosyltransferase activity (Otake et al., 1991). Glucosyltransferase is an extracellular enzyme that synthesizes EPS and provides a site on the tooth's surface for microbial colonization (Ren et al., 2016). Without these

extracellular enzymes, mutants of *S. mutans* are not cariogenic, which means they will not cause tooth decay (Devulapalle et al., 2001). Thus, EGCG plays an important role in the prevention of tooth caries because it has effectively inhibited glucosyltransferase (Hattori et al., 1990). Adding tea polyphenols (EGCG) to a diet has been shown to greatly decrease cavities in rats (Sakananka et al., 1986). The tolerable safe upper intake levels for EGCG can be up to 1000 mg daily (Miyazawa et al., 2000). One investigation indicated that participants consuming up to 1000 mg of EGCG daily for 3 months continuously did not show any differences on the serum biochemical analysis; they also did not have any sicknesses. Another study indicated that subjects consuming up to 900 mg of EGCG daily for 12 weeks showed no negative effects on the serum biochemical analysis (Nagao et al., 2005). There are other types of foods that can inhibit the growth of *S. mutans* (Nassar et al., 2011). However, a predominance of research demonstrates that green tea extract is one of the most effective components inhibiting *S. mutans* growth compared to other natural ingredients (Devulapalle et al., 2001; Nassar et al., 2011; Bender et al., 1986; Salli et al., 2016; Soderling et al., 2009). Most importantly, unlike xylitol and malititol, it has been discovered that the EGCG can be fully digested by the human body (Green et al., 2007; Chacko et al., 2010). Several studies have indicated that the EGCG can be digested by both human's and animal's digestive systems properly, in addition, several studies even indicated that EGCG could be used to treat diarrhea and to the knowledge of this researcher, no deleterious ingestive effect has been reported (Bora et al., 2017; McKay et al., 2002; Wu et al., 2003; Green et al., 2007). For example, when compared to the molecular structures of the tea polyphenols and sugar alcohols, xylitol has less OH. Furthermore, both xylitol

and maltitol have no C and O in their molecular structures as shown in **Figure 1** and **Figure 2**.

Chocolate is one the most popular snacks in the world. Recent research has indicated that dark chocolate has many benefits for human health, therefore the demand of dark chocolate has increased (Duarte et al., 2016). However, chocolate has also been considered a cavity causing food because of its high sugar content, which varies by food industry and the content of the cacao powder and cacao butter (Vasanthakumar et al., 2016). Sugar content can range from 0 grams to 40 grams per serving (**Table 8**) (Duarte et al., 2016; Orsa et al., 2012). There is a strong correlation between chocolate consumption and dental cavities, because several studies indicated that the increase amount of chocolate consumption induced the growth of dental cavities (Cevallos et al., 2015; Sadeghpour, 2015; Nirmala et al., 2016). Cevallos et al. along with other researchers discovered that after 10 minutes of chocolate intake, the pH level in the mouth significantly decreased. When the pH level decreased the mouth became more acid and this led to the demineralization of the tooth enamel and caused dental cavities. Furthermore, the researchers discovered that the subjects with a higher acid level of pH in the mouth were within the higher DMFS group. More importantly, the DMFS of the subjects raised when they increased the consumption of chocolate. Researchers even indicated that dark chocolates were more cariogenic than other types of chocolates, because according to pH level, dark chocolate was even more acid than the milk chocolate (Nirmala et al., 2016).

There were many studies suggesting that green tea polyphenol was effective in reducing *S. mutans* (Hirasawa et al., 2006; Mankovskaia et al., 2013; Hattori et al.,

1990; Miyazawa et al., 2000). However, there was limited research on how green tea polyphenols added in the food could inhibit the growth of *S. mutans* biofilm. Furthermore, several researchers indicated that chocolate was one of cariogenic food that caused cavities and this continues to be a debate within the scientific community. Therefore, the objectives of this study are 1) whether adding EGCG or EGCG-S, or P-EGCG in the 60% dark chocolate could inhibit the formation of *S. mutans* biofilm, 2) whether adding EGCG or EGCG-S, or P-EGCG in the 70% dark chocolate could inhibit the formation of *S. mutans* biofilm, 3) how much tea polyphenol needed to add to the dark chocolate to inhibit the growth of *S. mutans*, and 4) which of the three tea polyphenols is the most effective of inhibit the growth of *S. mutans*. Dark chocolate was produced and tea polyphenols, artificial saliva, and *S. mutans* were added in laboratory condition to discover the inhibition rate of the tea extract, as follows.

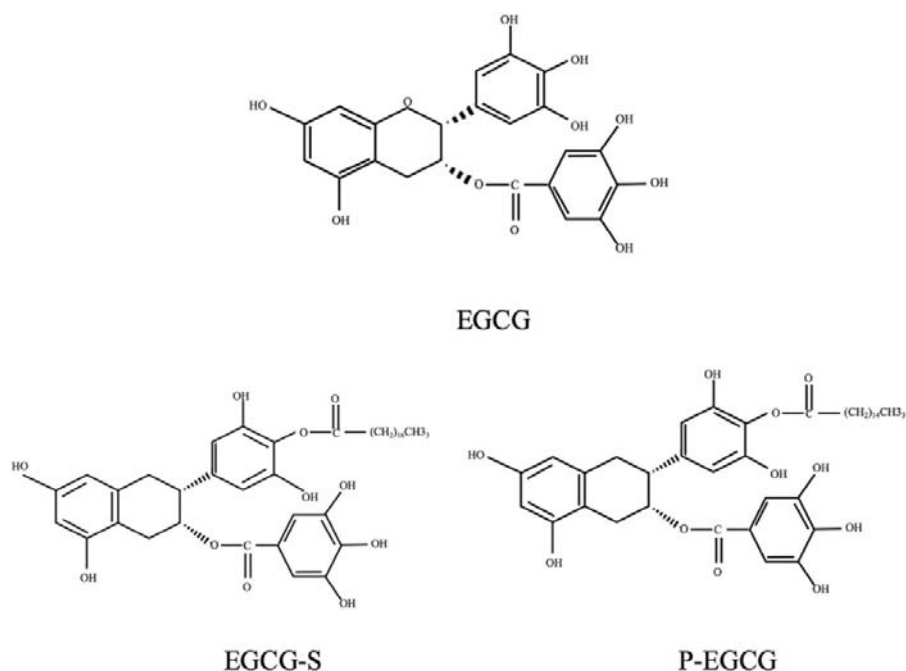


Figure 1. Molecular structures of EGCG, EGCG-S, and P-EGCG

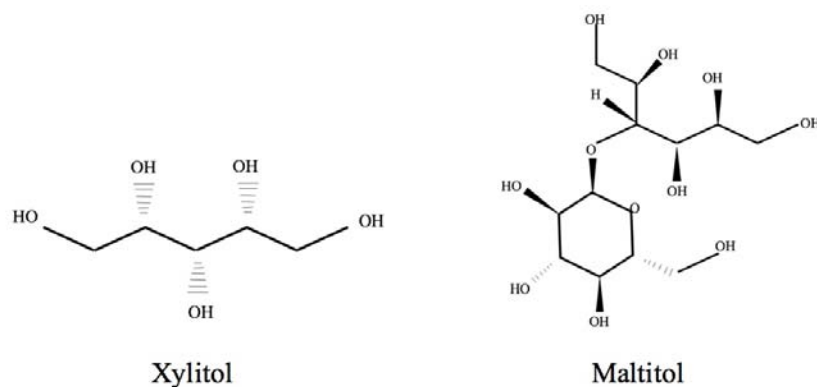


Figure 2. Molecular structures of xylitol and maltitol

2. Hypothesis

a. Hypothesis 1

H1: Adding EGCG or EGCG-S, or P-EGCG into dark chocolate inhibits the growth of *S. mutans*.

H0: Adding EGCG or EGCG-S, or P-EGCG into dark chocolate has no effect on the growth of *S. mutans*.

b. Hypothesis 2

H1: Tea polyphenols (EGCG, EGCG-S, and P-EGCG) inhibit the growth of *S. mutans* with different concentrations of sucrose in the artificial saliva.

H0: Tea polyphenols (EGCG, EGCG-S, and P-EGCG) has no effect on the growth of *S. mutans* with different concentrations of sucrose in the artificial saliva.

3. Materials and methods

3.1. Test bacterium, chemicals, and growth condition

S. mutans was maintained and grown on nutrient agar plates. Stock plates were regrown routinely to ensure the *S. mutans* effectiveness. Contaminated plates were eliminated at the start of the experiment. Epigallocatechin gallate (EGCG), palmitoyl-epigallocatechin gallate (P-EGCG) and epigallocatechin-3-gallate-stearate (EGCG-S) were purchased from Camellix LCC, (Augusta, GA, USA). The formula of the stock concentration of EGCG-S was 2.5mg/mL=0.0025g tea EGCG-S + 1mL of ethanol.

The Nutrient Agar was purchased from Difco™. The preparation of Nutrient Agar plate was using 30 grams of medium and 1 liter of deionized water and were used to prepare the nutrient agar plates. Then, the mixture was autoclaved at 121°C for 25 minutes. Next, the mixture was poured into individual sterile petri dishes and left to solidify. The Nutrient Broth was purchased from Difco™. The 30grams of medium and 1 liter of deionized water were put into a flask and mixed thoroughly till the medium was dissolved completely. Then, the solution was autoclaved at 121°C for 25 minutes. After autoclaving, the solution was cooled for 30 minutes.

The artificial saliva was prepared according to the Macknight-Hane and Whitford, 1992 formula. It contained Methyl-p-hydroxybenzoate, sodium carboxymethyl cellulose, KCl, MgCl₂ · 6H₂O, CaCl₂ · 2H₂O, K₂HPO₄, and KH₂PO₄.

3.2. *Culturing bacteria*

Streptococcus mutans was maintained and grown on nutrient agar plates. The stock plates were stored in a refrigerator at 4°C for future experimental usage. Stock plates were regrown routinely to ensure the *Streptococcus mutans* effectiveness. Before each experiment, if there was any contamination, the bacteria were eliminated.

a. Bacteria isolation

The stock culture plates were isolated using a continuous streaking method (**SUPPLEMENTARY APPENDIX - Figure 1**) on a nutrient agar plate, then it required an overnight incubation at 37°C. After incubation, the plates were wrapped in para-film and stored in the refrigerator at 4°C for next experiments.

b. Overnight culture

The overnight culture was prepared by adding 6mL of nutrient broth into sterile glass test tubes. A day before the experiment, the overnight culture should be prepared. Preparation of an overnight cultures was using a sterile cotton swab to streak a small portion of *Streptococcus mutans* off from the stock plate and mixed into the nutrient broth. Each overnight culture was labeled with date in which it was culture and the name of the bacteria. Then the cultures were incubated overnight at 37°C.

3.3. Simple stain of *S. mutans*

The purpose of simple stain was to have the purity check of *S. mutans*. A wire loop was heated with Bunsen burner until it turns orange color. After the wire loop was cooled down, using the wire loop to swipe some bacteria out from the plate and placed onto a sterile glass slide. Then a drop of crystal violatate was placed on top of the smear, and cover slip was place on top of the dye. The sample was observed under oil immersion at 400x magnification.

3.4. Dark chocolate preparation

The USDA does not provide an official standard for the preparation of dark chocolate. The 60% and 70% cacao content was determined by previous research and by the assessment of the amount of this ingredient commonly used in dark chocolate (**Table 8**) (Orsa et al., 2012). The 60% dark chocolate contained 35% of cacao powder, 25% of cocoa butter, 37% of sucrose, and 3% of the tea polyphenols (EGCG, EGCG-S, or P-EGCG). The 70% dark chocolate contained 40% of cacao powder, 30% of cocoa butter, 27% of sucrose, and 3% of the tea polyphenols (EGCG, EGCG-S, or P-EGCG). The control group of the 60% dark chocolate contained 35% of cacao powder, 25% of cocoa butter, 37% of sucrose, and 3% of water. The control group of the 70% dark chocolate contained 40% of cacao powder, 30% of cocoa butter, 27% of sucrose, and 3% of the water.

The raw cocoa beans were purchased from Santa Barbara Chocolate Co. The cocoa beans were washed. After drying, the cocoa beans were roasted at 121°C (250°F) for 25 minutes and the brown skin was removed from the cocoa beans. These

cocoa beans were ground into cacao powder. The raw and pure cocoa butter was purchased from North Oak Valley.

The cacao powder and cocoa butter were mixed together and heated until dissolved. Then the sucrose was added. After cooling, tea polyphenol was added to the chocolate. Stored the dark chocolate was not stored in the refrigerator for 24 hours until it become fully solid form.

3.5. Colony forming unit assay

The different percentage of sucrose was added with *S. mutans* grew in artificial saliva. These *S. mutans* was treated with EGCG, P-EGCG, or EGCG-S to identify whether these three green tea polyphenols (concentration 250ug/mL) could inhibit the growth. These preliminary studied was to understand the effectiveness of inhibition before adding these three green tea polyphenols to the dark chocolate.

The overnight culture of *S. mutans* with nutrient broth was diluted to 0.1 at OD_{600nm}. This overnight cultures of *S. mutans* were added with 0%, 1% or 5% of sucrose concentration of artificial saliva. This samples were treated with EGCG, EGCG-S, or P-EGCG at room temperature for 2, 4, and 24 hours. The control sample were treated with deionized water (DI H₂O). After 24-hour treatment with 50ug/ml of EGCG, EGCG-S, and P-EGCG, then, the serial dilutions were applied and plated on nutrient agar plates and incubated for 24 hours in the incubator at 37°C. After 24 hours of incubation, the colonies of *S. mutans* on the plates was counted as shown in **SUPPLEMENTARY APPENDIX - Figure 4**. It is used to evaluate the effect of time on the antimicrobial activity of these tea polyphenols. This experiment was

repeated three times and performed on separate days. The percentage of inhibition was calculated to determine whether the EGCG, EGCG-S, or P-EGCG could inhibit the growth of *S. mutans*. The following equation is used to calculate the percentage of inhibition:

$$\% \text{ of Inhibition} = \left(\frac{CFU_{control} - CFU_{treated}}{CFU_{control}} \right) \times 100.$$

The dark chocolate was melted at 36°C (96.8°F) into liquid form and added with artificial saliva (Weyland, 1998; Vasanthakumar et al., 2016). The proportion of dark chocolate and artificial saliva was based on the result of disk diffusion method experiment (**Table 5** and **Table 6**). The overnight culture of *S. mutans* with nutrient broth was diluted to 0.1 at OD_{600nm}. This overnight cultures of *S. mutans* were added with dark chocolate at room temperature for 2 hours. After 2 hours, the serial dilutions were applied and plated on nutrient agar plates and incubated for 24 hours in the incubator at 37°C. After 24 hours of incubation, the colonies of *S. mutans* on the plates was counted. Then, the CFU were calculated based on the colonies. This experiment was repeated three times and performed on separate days. The percentage of inhibition was calculated to determine whether the dark chocolate has effect of inhibit the growth of *S. mutans*.

3.6. Monitoring the growth of Streptococcus mutans

A standard growth curve is applied for monitoring the growth of *Streptococcus mutans* in different percentage of sugar content. The different percentage of sucrose concentrations are 0.1%, 0.5%, 1%, and 5%. First, the overnight culture of *Streptococcus mutans* with nutrient broth was diluted to 0.1 at OD_{600nm} (optical

density at 600nm). Then, this culture was incubated at the incubator at 37°C. Reading were recorded at every 2 hours at OD_{600nm} for 8 hours and the last reading was taken after 24 hours. This experiment was repeated for three times and the average of the reading was recorded. The standard deviation (SD) was used for this experiment.

3.7. The disk diffusion method

The disk diffusion method determined how much tea polyphenols should be added to the chocolate to inhibit the growth of *S. mutans*. Furthermore, it could determine the proportion between dark chocolate, artificial saliva, and P-EGCG that could inhibit the growth of *S. mutans*.

An overnight culture of *S. mutans* was prepared. The plates were divided into 4 sections and each section was labeled based on the treatment to be used in that section as shown in **SUPPLEMENTARY APPENDIX - Figure 3**. The cotton swap was used to perform continuous streaking method to swap the *S. mutans* onto the nutrient agar plates. Eight sterile tubes were prepared and filled with the solutions according to **Table 1**. The dark chocolate was melted into liquid form at 36°C (96.8°F). The solutions contained 60% dark chocolate and artificial saliva. Sterilized forceps were used to place each disk in the section of the nutrient agar plate and pressed gently. The forceps were flamed with a Bunsen burner in between the placement of each disk. Forty-five ul of the solution was obtained from each tube and placed on each disk. Five ul of H₂O was added to the disk as the control group and 5ul of P-EGCG (concentration = 250ug/ml) were added to the disk as the experimental group, as shown in **SUPPLEMENTARY APPENDIX - Figure 3**. These plates were incubated

overnight. After the incubation, the zone of inhibition was measured. The method of measuring the zone of inhibition was shown in **SUPPLEMENTARY APPENDIX – Figure 3**.

The zone of inhibition (ZOI) was measured of each plate after 24 hours and 48 hours. The ZOI was measured from the end of the clearing zone to the edge of the disk as shown in **SUPPLEMENTARY APPENDIX – Figure 3**. ZOI was measured in millimeters. The average of 4 measurements per disk was recorded. This experiment was repeated three times and performed on separate days.

Table 1. The concentration of artificial saliva with dark chocolate	
100%	1g of dark chocolate + 0mL of artificial saliva
75%	0.75g of dark chocolate + 0.25mL of artificial saliva
50%	0.5g of dark chocolate + 0.5mL of artificial saliva
25%	0.25g of dark chocolate + 0.75mL of artificial saliva

Table 1. The proportion of artificial saliva together with 60% dark chocolate, simulates different amounts of dark chocolate that remained in the mouth.

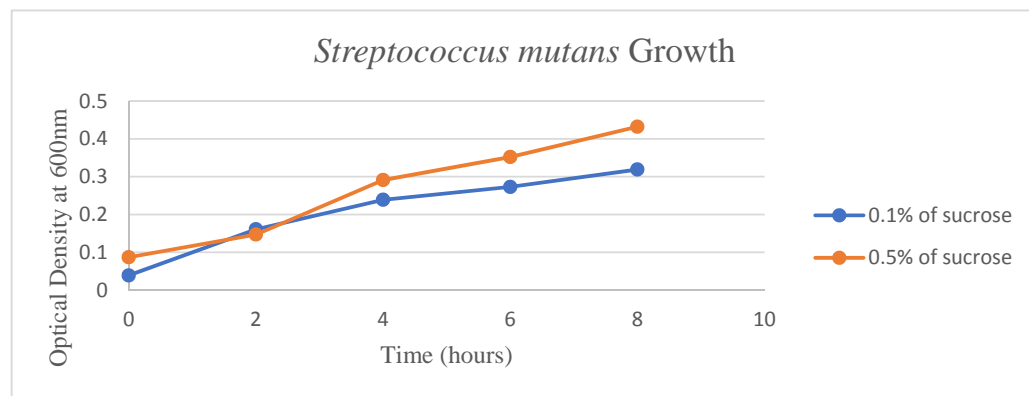
3.8. Statistical analysis

All experiments were performed in triplicate at separate times. Differences between the control group and experimental groups were analyzed by SPSS. ANOVA test was performed and the significance value was set at a p value of <0.05.

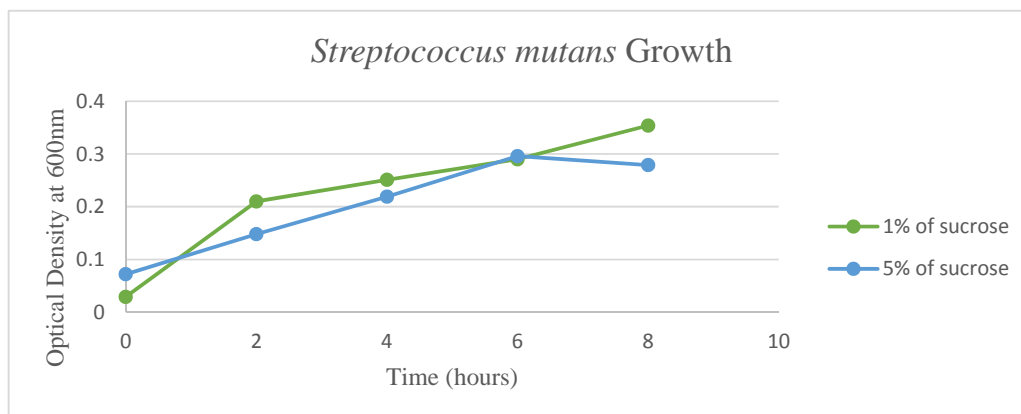
4. Results

4.1. Monitoring the growth of *Streptococcus mutans*

The growth of *S. mutans* in different concentration of sucrose was observed for a period of 8 hours. The absorbance was read every 2 hours at an optical density of 600nm. In **Figure 3**, a standard growth curve displays an exponential growth of *S. mutans* in different concentration of sucrose in artificial saliva for 8 hours. The *S. mutans* culture was grown in nutrient broth with different concentration of sucrose. In **Figure 4**, a standard growth curve displays an exponential growth of *S. mutans* in different concentration of sucrose in nutrient broth for 8 hours. The standard growth curve indicated that sucrose could provide essential nutrient for *S. mutans* constantly for 8 hours, therefore the death phase did not occur during a period of 8 hours. Based of the equation $g = (\log_{10} N_1 - \log_{10} N_0) / \log_{10} 2$, showed that the generation time of *S. mutans* that growth in different concentration of sucrose is 8 hours.



(a)



(b)

Figure 3. Standard growth curve of *Streptococcus mutans* in artificial saliva with (a) 0.1% or 0.5% and (b) 1% or 5% of concentration of sucrose. The growth was read at optical density at 600nm using spectrophotometer. Samples were kept in an incubator at 37°C for the experiment.

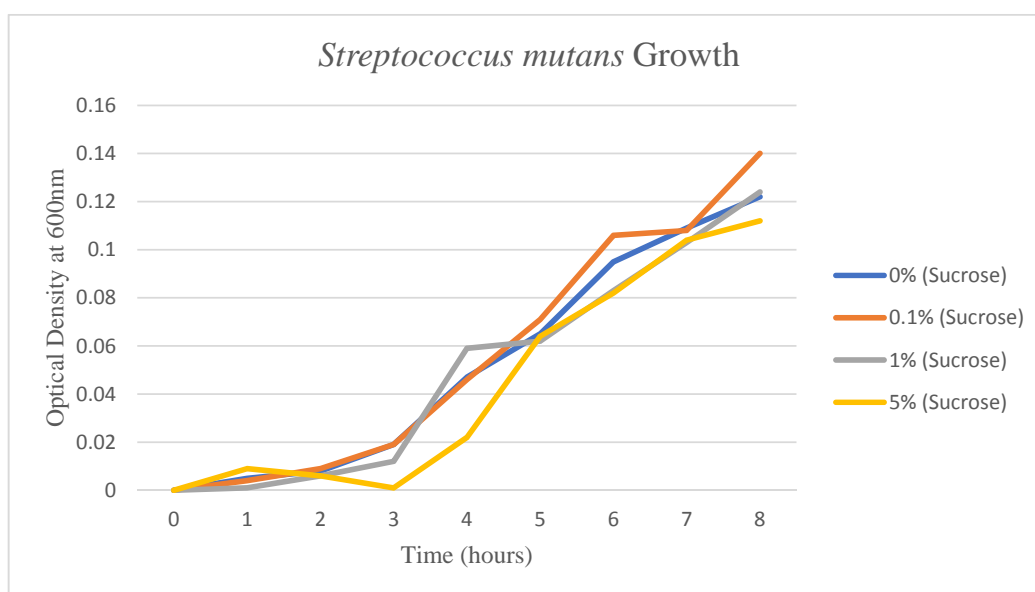


Figure 4. Standard growth curve of *Streptococcus mutans* in nutrient broth with 0.1%, 0.5%, 1%, or 5% of concentration of sucrose. The growth was read at optical density at 600nm using spectrophotometer. Samples were kept in an incubator at 37°C for the experiment.

4.2. Identification the growth of Streptococcus mutans

The simple stain was performed to identify the morphology of *S. mutans*. In **SUPPLEMENTARY APPENDIX - Figure 4**, a simple stain of *S. mutans* showed that the bacteria are circular and cocci shaped that are connected like the chains.

4.3. The three tea polyphenols inhibit the growth of S. mutans growth in different concentration of sucrose.

The three tea polyphenols (EGCG, P-EGCG, EGCG-S) inhibited the *in vitro* growth of *S. mutans* in artificial saliva with different concentrations of sucrose. In order to determine the effect of the three tea polyphenols on the *S. mutans*, the deionized water was added to the artificial saliva as the control group. Compared to the control group, the inhibition rates of *S. mutans* with 0% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively, $6.01\% \pm 41.4\%$, $77.8\% \pm 0.4\%$, and $81.8\% \pm 11.7\%$ after 2 hours (**Table 2**). After 4 hours, the inhibition rates of *S. mutans* with 0% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively, $81.7\% \pm 10.1\%$, $94.7\% \pm 0.3\%$, and $97.9\% \pm 0.3\%$ (**Table 2**). After 24 hours, the inhibition rates of *S. mutans* with 0% sucrose and EGCG, EGCG-S, and P-EGCG were $-162.8\% \pm 4.0\%$, $94.6\% \pm 1.4\%$, and $99.4\% \pm 0.3\%$, respectively (**Table 2**). Both EGCG-S and P-EGCG significantly inhibited the *S. mutans* in 2, 4, and 24 hours (**Figure 5**, $p < 0.05$).

Compared to the control group, the inhibition rates of *S. mutans* with 1% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively, $61.2\% \pm 11.7\%$, $93.1\% \pm 0.1\%$, and $86.9\% \pm 5.2\%$ after 2 hours (**Table 2**). After 4 hours, the inhibition rates of

S. mutans with 1% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively, $75.7\% \pm 5.8\%$, $96.4\% \pm 0.2\%$, and $96.0\% \pm 0.2\%$ (**Table 2**). After 24 hours, the inhibition rates of *S. mutans* with 1% sucrose and EGCG, EGCG-S, and P-EGCG were $-30.3\% \pm 6.8\%$, $97.45\% \pm 0.6\%$, and $99.6\% \pm 0.1\%$, respectively (**Table 2**). Both EGCG-S and P-EGCG significantly inhibited the *S. mutans* in 2, 4, and 24 hours (**Figure 5**, $p < 0.05$).

Compared to the control group after 2 hours the inhibition rates of *S. mutans* with 5% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively, $31.3\% \pm 14.2\%$, $99.9\% \pm 0.03\%$, and $90.1\% \pm 9.8\%$ (**Table 2**). After 4 hours, the inhibition rates of *S. mutans* with 5% sucrose and EGCG, EGCG-S, and P-EGCG were $68.3\% \pm 2.4\%$, $99.7\% \pm 0.04\%$, and $99.3\% \pm 0.2\%$, respectively. (**Table 2**). After 24 hours, the inhibition rates of *S. mutans* with 5% sucrose and EGCG, EGCG-S, and P-EGCG were, respectively $-27.9\% \pm 11.5\%$, $99.9\% \pm 0.1\%$, and $99.4\% \pm 0.1\%$ (**Table 2**). Both EGCG-S and P-EGCG significantly inhibited the 5% sucrose with *S. mutans* in 2,4, and 24 hours (**Figure 5**, $p < 0.05$).

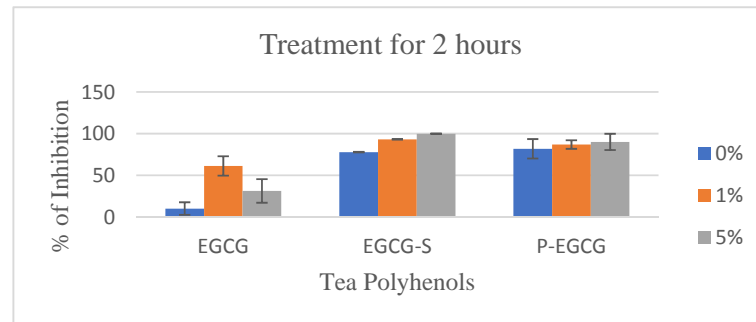
Table 2 – Percentage of inhibition

0% of sucrose	2 hours, n=3	4 hours, n=3	24 hours, n=3
Control (DI H ₂ O)	0%	0%	0%
EGCG	10% \pm 7.5%	82% \pm 10.1%	-163% \pm 4.0%
EGCG-S	78% \pm 0.4%	95% \pm 0.3%	95% \pm 1.4%
P-EGCG	82% \pm 11.7%	98% \pm 0.3%	100% \pm 0.3%
1% of sucrose	2 hours, n=3	4 hours, n=3	24 hours, n=3
Control (DI H ₂ O)	0%	0%	0%
EGCG	61 % \pm 11.7%	76% \pm 5.8%	-30% \pm 6.8 %
EGCG-S	93% \pm 0.1%	96% \pm 0.2%	97% \pm 0.6%
P-EGCG	87% \pm 5.2%	96% \pm 0.2%	100% \pm 0.1 %
5% of sucrose	2 hours, n=3	4 hours, n=3	24 hours, n=3
Control (DI H ₂ O)	0%	0%	0%
EGCG	31% \pm 14.2%	68% \pm 2.4%	-28% \pm 11.5%
EGCG-S	100% \pm 0.03%	100% \pm 0.04%	100% \pm 0.1%
P-EGCG	90% \pm 9.8%	100% \pm 0.2%	99% \pm 0.1%

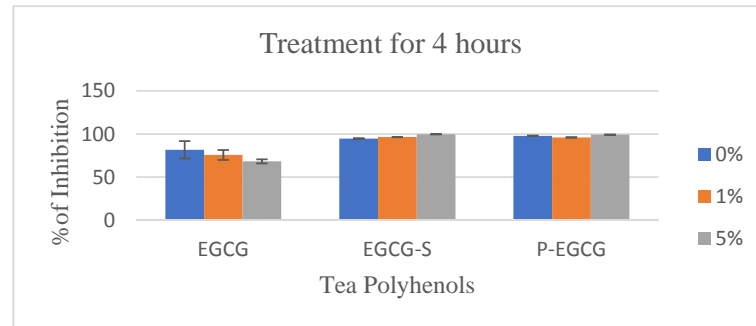
*Tea polyphenols concentration = 250ug/mL

Table 2. The percentage of inhibition was calculated from the colony forming unit for EGCG, EGCG-S, and P-EGCG at different concentrations of sucrose at different time intervals over 24 hours.

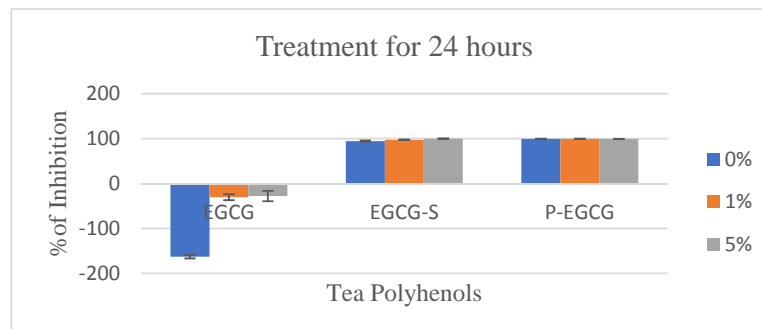
Figure 5 - Comparison of three types of Tea Polyphenols effect on *S. mutans* during different time



(a)



(b)



(c)

Figure 5. Effect of three types of tea polyphenols on *S. mutans* at different concentrations of sucrose. (a) treated for 2 hours ($n=9$); (b) treated for 4 hours ($n=9$); (c) treated for 24 hours ($n=9$). Blue bars represent the 0% of sucrose within artificial saliva, orange bars represent the 1% of sucrose within artificial saliva, and grey bars represent 5% of sucrose within artificial saliva. Statistical significance was determined by ANOVA.

4.4. The cacao butter enhances the growth of *S. mutans* and the cacao liquid has no effect on the growth of *S. mutans*

The cacao butter enhanced the *in vitro* growth of *S. mutans*. In order to determine the effect of cacao butter on the *S. mutans*, the *S. mutans* growth in the nutrient broth served as the control group. The colonies forming units of *S. mutans* growth in nutrient broth, deionized H₂O, and cacao butter are 33 ± 2.573 , 39 ± 2.641 , 229 ± 5.778 , respectively (**Table 3**, $p < 0.05$). Unlike cacao butter, the cacao liquid did not affect the growth of *S. mutans*. The *S. mutans* growth in the nutrient broth served as the control group. The colonies forming units of *S. mutans* growth in nutrient broth, deionized H₂O, and cacao liquid are 29 ± 1.563 , 12 ± 2.731 , 9 ± 2.876 , respectively (**Table 4**, $p < 0.05$). Based on **SUPPLEMENTARY APPENDIX - Figure 14**, the number of CFU of *S. mutans* growth in different medias are similar.

Table 3 - Colony forming units of *S. mutans* in cacao butter (n=3)

Media	<i>Streptococcus mutans</i> (CFU 10 ⁻⁵)
Nutrient broth	33 ± 2.573
Deionized H ₂ O	39 ± 3.641
Cacao butter	229 ± 5.778

Table 3. The number of colonies of *S. mutans* growth within cacao butter. Based on the CFU, cacao butter enhances the growth of *S. mutans*.

Table 4 - Colony forming units of *S. mutans* in cacao liquid (n=3)

Media	<i>Streptococcus mutans</i> (CFU 10 ⁻⁵)
Nutrient broth	29 ± 1.563
Deionized H ₂ O	12 ± 2.731
Cacao liquid	9 ± 2.876

Table 4. The number of colonies of *S. mutans* growth within cacao liquid. Based on the CFU, cacao liquid has no effect on the growth of *S. mutans*.

4.5 P-EGCG added with different concentration of chocolate within artificial saliva inhibit the *S. mutans*

The disk diffusion method determined how much P-EGCG needed to be added to the dark chocolate. P-EGCG added with different proportion of chocolate and artificial saliva created a different antibacterial result.

After 24 hours, the zone of inhibition of 100% concentration, 75% concentration, 50% concentration, and 25% concentration of dark chocolate within artificial saliva with treatment of P-EGCG were, respectively, $1.66\text{mm} \pm 1.15\text{mm}$, $1.16\text{mm} \pm 0.28\text{mm}$, $2.00\text{mm} \pm 0.90\text{mm}$, and $2.41\text{mm} \pm 1.37\text{mm}$ (**Table 5**). After 24 hours, the zone of inhibition of 100% concentration, 75% concentration, 50% concentration, and 25% concentration of dark chocolate within artificial saliva with treatment of DI H₂O do not have zone of inhibition. (**Table 5**).

After 48 hours, the zone of inhibition of 100% concentration, 75% concentration, 50% concentration, and 25% concentration of dark chocolate within artificial saliva with treatment of P-EGCG were, respectively, $1.91\text{mm} \pm 1.37\text{mm}$, $1.50\text{mm} \pm 0.43\text{mm}$, $2.66\text{mm} \pm 1.84\text{mm}$, and $2.41\text{mm} \pm 0.76\text{mm}$ (**Table 6**). After 48 hours, the zone of inhibition of 100% concentration, 75% concentration, 50% concentration, and 25% concentration of dark chocolate within artificial saliva with treatment of DI H₂O do not have zone of inhibition. (**Table 6**).

Table 5 - The zone of inhibition of P-EGCG disk diffusion tests for <i>S. mutans</i> (n=3)	
Concentration of dark chocolate within artificial saliva	P-EGCG (250ug/ml)
100%	1.66mm \pm 1.15mm
75%	1.16mm \pm 0.28mm
50%	2.00mm \pm 0.90mm
25%	2.41mm \pm 1.37mm

Table 5. The zone of inhibition (ZOI) was taken after 24 hours.

Table 6 - The zone of inhibition of P-EGCG disk diffusion tests for <i>S. mutans</i> (n=3)	
Concentration of dark chocolate within artificial saliva	P-EGCG (250ug/ml)
100%	1.91mm \pm 1.37mm
75%	1.50mm \pm 0.43mm
50%	2.66mm \pm 1.84mm
25%	2.41mm \pm 0.76mm

Table 6. The zone of inhibition (ZOI) was taken after 48 hours.

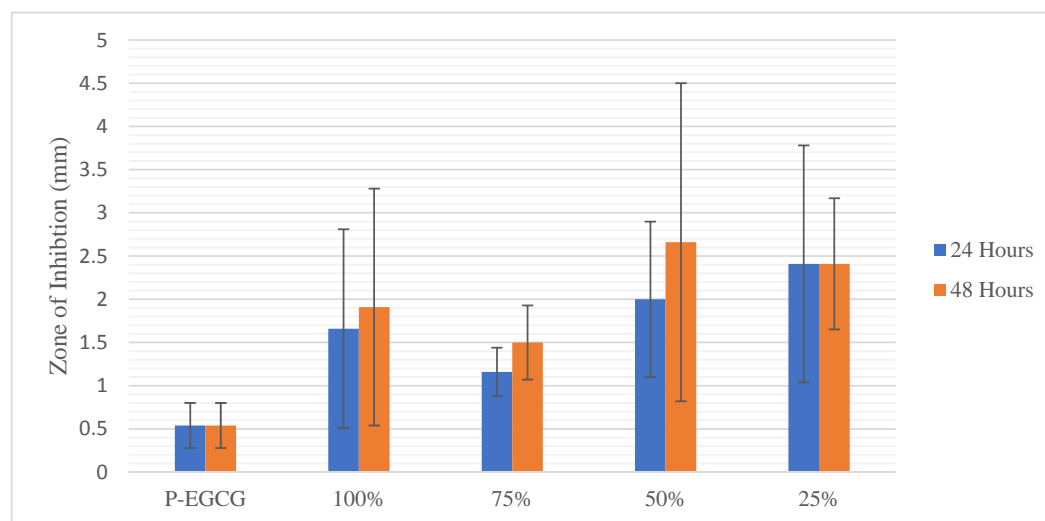


Figure 6. Effect of P-EGCG and P-EGCG with different proportions of dark chocolate within artificial saliva on *S. mutans* under 24 hours and 48 hours of treatment. The x-axis represents different concentrations of dark chocolate within artificial saliva. The blue bars represent the treatment for 24 hours and the orange bars represent the treatment for 48 hours (n=3).

4.6. The P-EGCG and EGCG-S inhibits the growth of *S. mutans* in the dark chocolate

The three tea polyphenols (EGCG, P-EGCG, EGCG-S) inhibited the *in vitro* growth of *S. mutans* in both 60% and 70% dark chocolate. In order to determine the effect of the three tea polyphenols on the *S. mutans*, dark chocolate without tea polyphenols which served as the control group.

The colony forming units of *S. mutans* treated with the 60% dark chocolate added with no tea polyphenols, EGCG, EGCG-S, and P-EGCG are 553 ± 19.69 , 244 ± 18.73 , 161 ± 15.6 , and 100.6 ± 17.7 (**Table 7**). The colony forming units of *S. mutans* treated with the 70% dark chocolate added with no tea polyphenols, EGCG, EGCG-S, and P-EGCG are 373 ± 27.5 , 251 ± 11.37 , 252 ± 3.60 , and 222.3 ± 8.08 (**Table 7**).

The EGCG, EGCG-S, and P-EGCG inhibited the *S. mutans* in 60% dark chocolate at inhibition rates of, respectively, $55.9\% \pm 5.04\%$, $70.9\% \pm 1.90\%$, and $81.7\% \pm 2.69\%$ (**Table 8**). Both EGCG-S and P-EGCG significantly inhibited the *S. mutans* compared to the 60% dark chocolate that had no tea polyphenols added (**Figure 7**, $p < 0.05$). The EGCG, EGCG-S, and P-EGCG inhibited the *S. mutans* in 70% dark chocolate at inhibition rates of $32.4\% \pm 5.85\%$, $32.5\% \pm 4.21\%$, and $40.2\% \pm 4.70\%$, respectively (**Table 8**). The P-EGCG significantly inhibited the *S. mutans* compared to the 70% dark chocolate that had no tea polyphenols added (**Figure 7**, $p < 0.05$).

Table 7 – Colony forming units of *S. mutans* in 60% and 70% dark chocolate

60% dark chocolate 1g/10ml of artificial saliva (n=3)					
	Repeating 1	Repeating 2	Repeating 3	Mean	Standard Deviation
Control	569	531	559	553	± 19.6977156
EGCG	229	265	238	244	± 18.734994
EGCG-S	169	143	171	161	± 15.6204993
P-EGCG	121	88	93	100.6	± 17.7857621

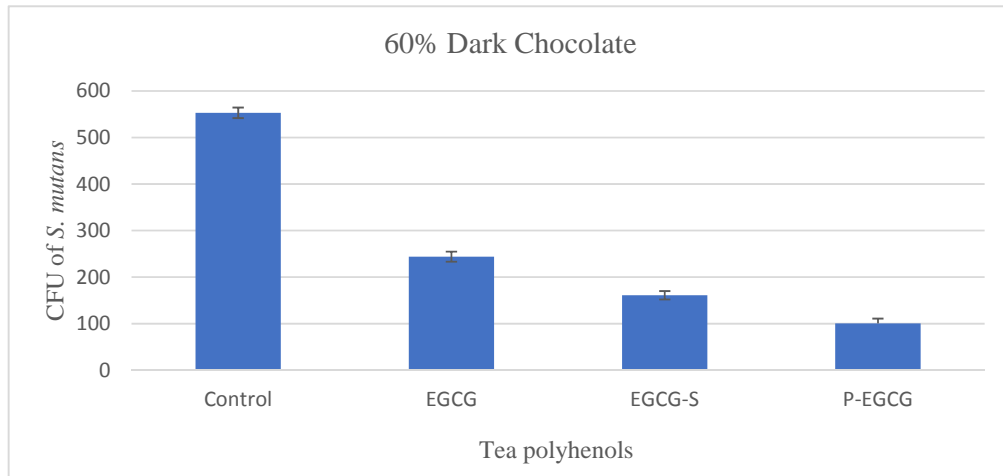
70% dark chocolate 1g/10ml of artificial saliva (n=3)					
	Repeating 1	Repeating 2	Repeating 3	Mean	Standard Deviation
Control	363	405	351	373	± 27.5922694
EGCG	264	248	242	251.3	± 11.3724814
EGCG-S	249	256	251	252	± 3.60555127
P-EGCG	231	221	215	222.3	± 8.08290376

Table 7. The number of colonies of *S. mutans* growth in 60% and 70% dark chocolate.

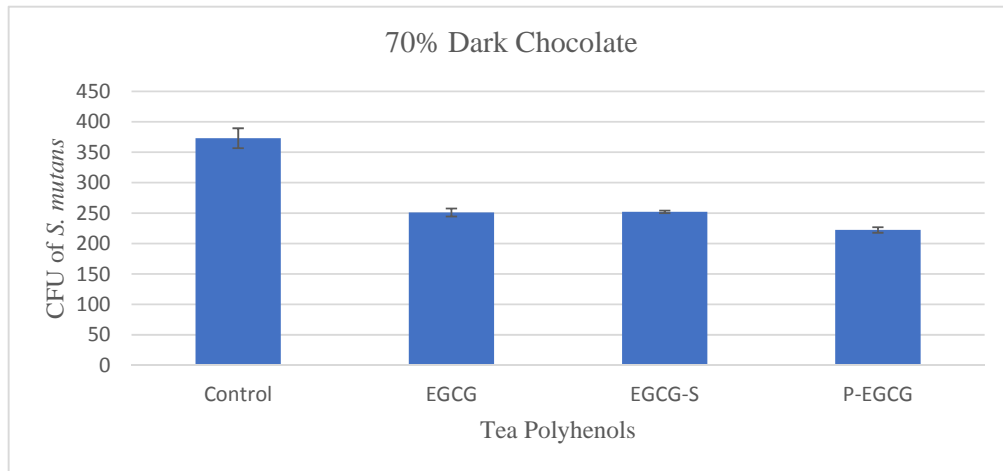
Table 8 – Percentage of inhibition

	60% dark chocolate (n=3)	70% dark chocolate (n=3)
Control	0	0
EGCG	56 ± 5.04	32 ± 5.85
EGCG-S	71 ± 1.90	33 ± 4.21
P-EGCG	82 ± 2.69	40 ± 4.70

Table 8. The percentage of inhibition was calculated from the colony forming unit for EGCG, EGCG-S, and P-EGCG at room temperature for 2 hours (n=6).

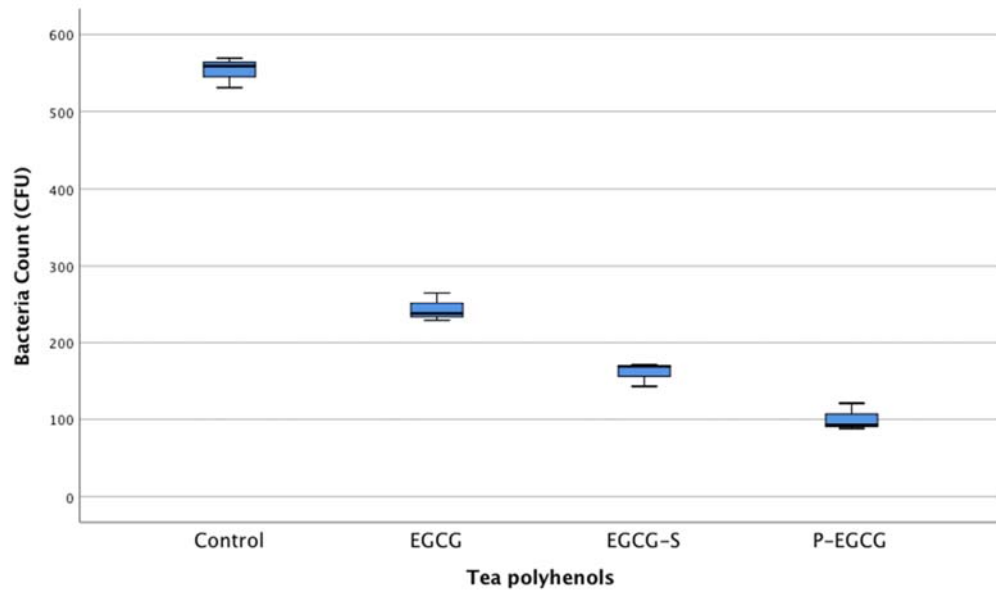


(a)



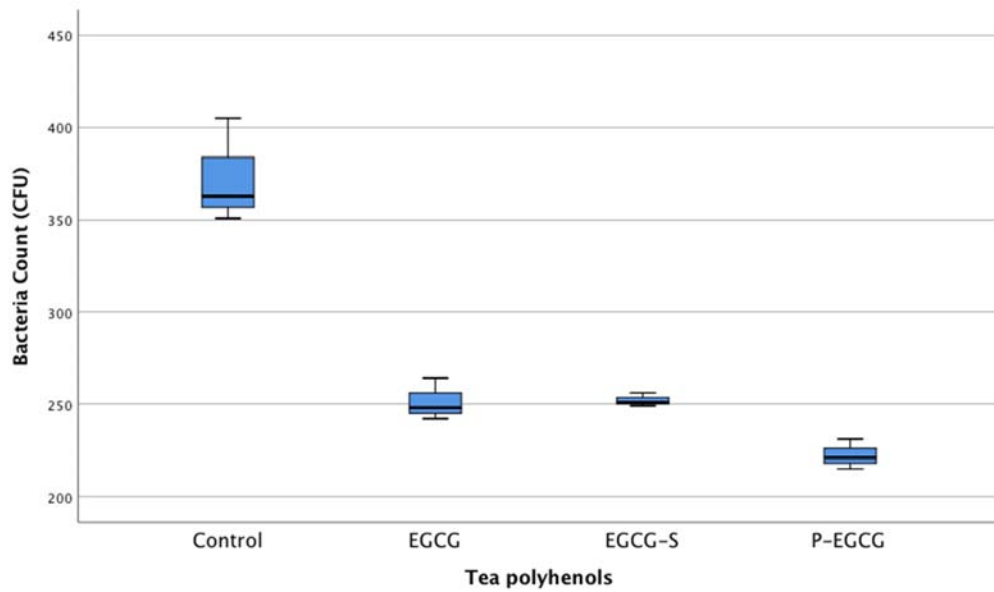
(b)

Figure 7. Effect of three types tea polyphenol on *S. mutans*. (a) 60% dark chocolate; (b) 70% dark chocolate ($n=6$). Statistical significance was determined by ANOVA ($p<0.05$).



$n=3$

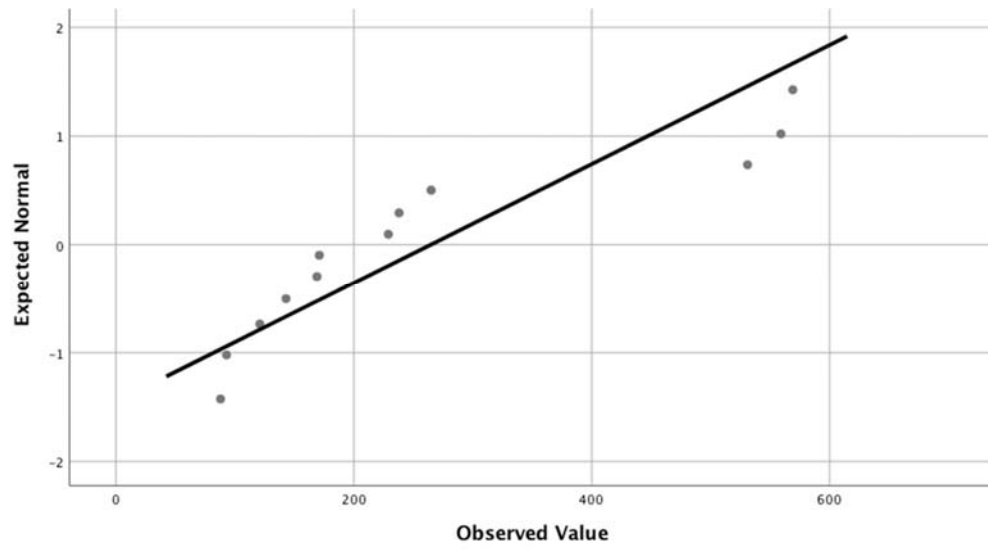
(a)



$n=3$

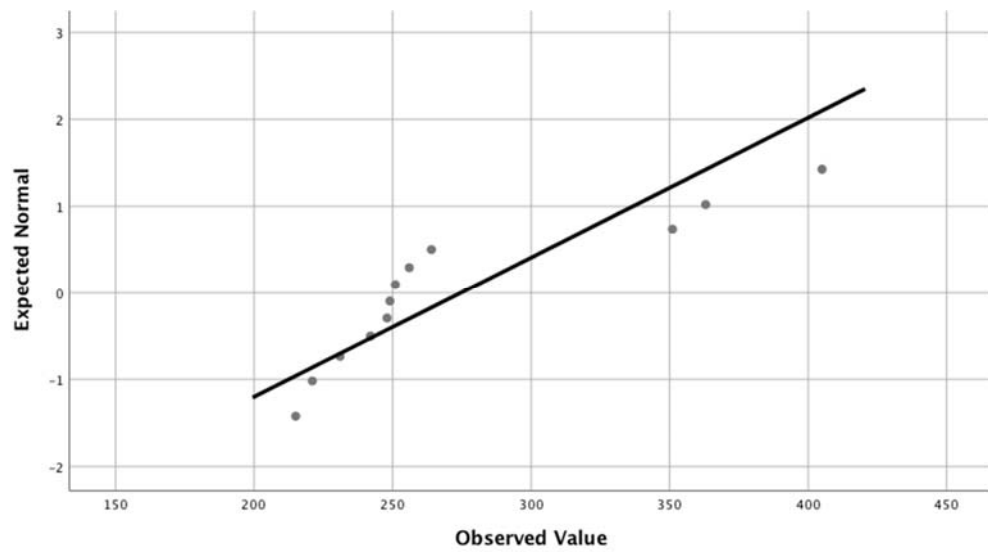
(b)

Figure 8. Effect of three tea polyphenols on *S. mutans*. (a) 60% dark chocolate; (b) 70% dark chocolate. Statistical significance was determined by ANOVA ($p<0.05$).



$n=3$

(a)



$n=3$

(b)

Figure 9. Normal Q-Q plot of effect of three tea polyphenols on *S. mutans*. (a) 60% dark chocolate; (b) 70% dark chocolate. Statistical significance was determined by ANOVA ($p < 0.05$).

5. Discussion

S. mutans are the most common bacteria in the dental plaque community (David et al., 2011). This bacteria synthesizes extracellular polysaccharides (EPS) from dietary sucrose, which could result in dental caries (Loesche, 1986; WHO, 2012; Forssten et al., 2010; Gupta et al., 2013). Furthermore, individual intake of high-sugar products is increasing in several developing countries (WHO, 2013), because many processed foods contain a high amount of simple carbohydrates (Morais et al., 2014). The affordability of the processed foods and lack of knowledge of eating healthy contribute to this rise.

Several studies have suggested green tea polyphenols are effective in reducing *S. mutans* (Hirasawa et al., 2006; Mankovskaia et al., 2013). In the present study, the highest inhibition rate of the tea polyphenols (concentration 250ug/mL) on *S. mutans* was up to $81\% \pm 10.1\%$. Furthermore, the inhibition rate of EGCG is not as effective as the other two types of tea polyphenols (EGCG-S and P-EGCG). Both EGCG-S and P-EGCG significantly inhibited the *S. mutans* for up to 24 hours with an average inhibition rate of 98%. This result agrees with the other studies that indicated EGCG is one of the green tea polyphenols that inhibit the growth of *S. mutans* (Kaur et al., 2014; Hirasawa et al., 2006; Mankovskaia et al. 2013).

The amount of residue of dark chocolate that remains in our mouths after human consumption is unclear. Therefore, different proportion of chocolate within the artificial saliva treated with tea polyphenol needed to be carried out in this research. When simulating oral conditions, **Table 5** and **Table 6** showed that when P-EGCG was added to dark chocolate and artificial saliva, depending on the different

proportions, the zone of inhibition was revealed. There was no zone of inhibition for the control group, H₂O treatment. In contrast, the zone of inhibition of P-EGCG ranged from 1.16mm \pm 0.28mm to 2.00mm \pm 0.90mm. The zone of inhibition of the lower proportion of dark chocolate within artificial saliva was higher than the denser proportion of dark chocolate within artificial saliva. This result also showed that without artificial saliva, the P-EGCG added to the dark chocolate could have inhibited the growth of *S. mutans*. **Table 5** and **Table 6** indicated that there needed to be at least 0.5% of P-EGCG added to the dark chocolate to inhibit the growth of *S. mutans*. The researchers also discovered that after 48 hours, the control group (treatment with H₂O), the denser proportion of dark chocolate within artificial saliva, enhanced the growth of *S. mutans* (**SUPPLEMENTARY APPENDIX - Figure 16B** and **Figure 20B**). This result suggested that higher proportion of dark chocolate within saliva increased the chance of causing dental caries.

Dark chocolate contains high amounts of sugar and is considered a cavity causing food (Gupta et al., 2013; Hirasawa et al. 2006). In the present research, the two types of laboratory produced dark chocolate contained 60% and 70% cacao. The 60% dark chocolate contained 37% sucrose, and the 70% dark chocolate contained 27% sucrose. This range of sugar content was based on an average range of different brands of dark chocolate in the market (**Table 8**). The 60% dark chocolate presented the worst condition for testing the effectiveness of the green tea polyphenols because it contained 37% sucrose. The laboratory produced dark chocolates had the same percentage of sucrose to commercially made Ghirardelli Dark Chocolate, which is 36% sugar. In the present study, when comparing the 60% dark chocolate with the 70% dark

chocolate, different amounts of sucrose contained in the laboratory produced dark chocolates, revealed that the amount of sucrose does affect the growth of *S. mutans*. Based on the **Table 7**, the bacteria colonies of the 70% dark chocolate are fewer than the bacteria colonies of the 60% dark chocolate. This is possibly because the sucrose in the 70% dark chocolate was 10% less than the 60% dark chocolate, which supports the previous research that indicating sucrose enhanced the growth of *S. mutan* (Oda et al., 2015; Loesche, 1986). In the present study, the researchers discovered that all three tea polyphenols added to the dark chocolate varieties could inhibit the growth of *S. mutans*, especially P-EGCG. The inhibition rate of the P-EGCG added to the 60% dark chocolate was $81\% \pm 2.69\%$. The inhibition rate of P-EGCG added to the 70% dark chocolate was $40\% \pm 4.70\%$, which indicated that since 70% dark chocolate had a higher content of sucrose, therefore the inhibition rate was lower than the 60% dark chocolate.

All experiments were performed on separate days and the same experimental method was performed at least three times on different days. Therefore, the standard deviations and correlations were only among limited samples and in turn were a limitation. The limitation of this research study was that each of the experiments were only performed three times because most of the experimental methods, such as colony forming unit assay and disk diffusion, were time-consuming and costly. The other limitation of this study was that the dark chocolates that were on the market were not tested. Therefore, whether tea polyphenols added to these dark chocolates were still effective on inhibiting the growth of *S. mutans*, remains unclear.

Table 8 – Sugar content of different brands of dark chocolates	
Dark chocolates (100 grams)	Sugar content (Per 100 grams)
Ghirardelli Dark Chocolate 60% Cacao	36 grams
Ghirardelli Dark Chocolate 72% Cacao	26 grams
Ghirardelli Dark Chocolate 86% Cacao	11 grams
Lindt Dark Chocolate 70% Cocoa	30 grams
Lindt Dark Chocolate 85% Cocoa	12.5 grams
Lindt Dark Chocolate 90% Cocoa	7.5 grams
Green & Black's Dark Chocolate 70% Cacao	27.5 grams
Green & Black's Dark Chocolate 85% Cacao	12.5 grams

Table 8. The sugar content of different brands of dark chocolates on the market of the United States of America.

6. Implication and Application

The results from this study reveal several practical applications that would contribute to the food industry. According to the 2015 to 2017 statistic from World Health Organization, 60-90% of children and nearly 100% of adults have dental cavities around the world, however, these rates remain steady and have not declined since 2015. In addition, there is a strong correlation between dark chocolate consumption and dental cavities (Sadeghpour, 2015; Nirmala et al., 2016), although, there are no previous studies that have addressed this problem. Previous studies discovered that the cavity rate increases because individuals consume high sugary processed food and do not practice good oral hygiene behavior (Maharani et al., 2017;

Achmad., 2016). Therefore, a popular processed food, that does not cause dental cavities, such as dark chocolate, would be beneficial for these groups of individuals.

Some chocolate companies are using sugar substitute to replace sugar in their chocolate products in order to reduce the calories and the rise of dental caries. For example, Hersey uses maltitol as a sugar substitute, but maltitol has been discovered it can cause laxative effect and sometimes can cause stomach bloating and gas (Yadav et al., 2014; Chukwuma et al., 2016). Previous studies have discovered tea polyphenols could effectively prevent dental caries and digestion problems (Hirasawa et al., 2006; Mankovskaia et al., 2013; Green et al., 2007). However, not many studies have addressed whether these tea polyphenols could be added to food and still remains effective.

Although a limitation of the present findings was a lack of generalized data, this study still suggests that the tea polyphenols could be used as a food additive for dark chocolate and decrease the chance of having dental cavities. The chocolate industries could add the tea polyphenols into their chocolate product with regular sugar instead of sugar substitute and still reduce the cavity rate and calories without sacrificing taste. In addition, the outcome of this research shows it would be beneficial for individuals by decreasing their chance of having dental cavities when they consume chocolate.

7. Conclusion

The main purpose of this study was to discover whether adding tea polyphenols to the dark chocolate would inhibit the growth of *S. mutan* and dental cavities. The more sugar exposed to the *S. mutans*, the higher the likelihood that cavities will occur (Gupta

et al., 2013). In the present study, the researchers concluded that the tea polyphenols added to the dark chocolate could inhibit *S. mutans*. The tea polyphenols are effective in reducing *S. mutans* growth in different concentrations of sucrose. The highest inhibition rate of tea polyphenols (concentration = 250 ug/ml) on different concentrations of sucrose was up to 100% \pm 0.1%. This research also indicated that EGCG was not as effective as the other two types of tea polyphenols and indeed both EGCG-S and P-EGCG proved that the inhibition rate was up for 24 hours with an average inhibition rate of 98%.

The disk diffusion method indicated that there needed to be at least 0.5% of P-EGCG (Concentration =250ug/mL) added to the dark chocolate to inhibit the growth of *S. mutans*. The zone of inhibition of the lower proportion of dark chocolate within artificial saliva was higher than the denser proportion of dark chocolate within artificial saliva. Based on the findings in the present research, green tea polyphenols are potentially effective in reducing *S. mutans*, because EGCG-S inhibited *S. mutans* significantly in the 60% dark chocolate with an inhibition rate of 71% \pm 1.90. Furthermore, P-EGCG significantly inhibited *S. mutans* in the 60% dark chocolate with inhibition rate of 82% \pm 2.69% ($P<0.05$). This study suggests that these green tea polyphenols have the potential to be used as a food additive for dark chocolate.

The limitation concerning the findings is that it was based on limited data, therefore, the results are not conclusively proven. The other limitation of this research is that the researchers did not test the dark chocolate sold in supermarkets. Instead the researchers used the chocolate created in the lab. Therefore, the researchers cannot conclude that adding the tea polyphenols to these chocolates will be effective. Furthermore, a

multi-sensory evaluation of the dark chocolate with tea polyphenols should be carried out in the future. The shelf-life of the dark chocolate added with the tea polyphenols need further study.

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SUPPLEMENTARY APPENDIX

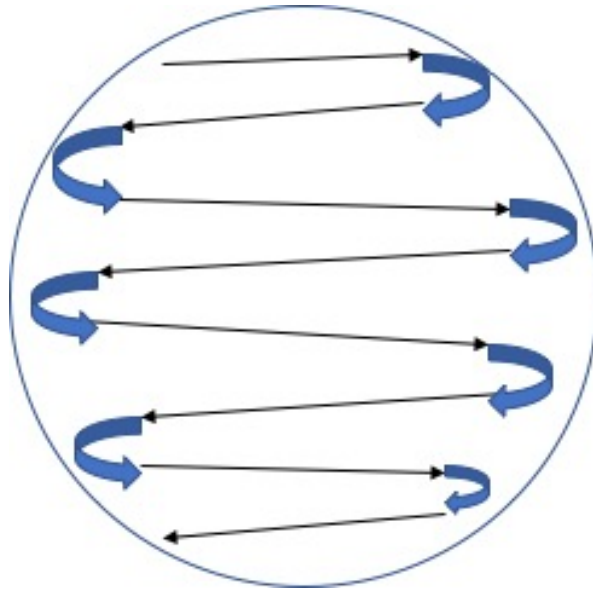


Figure 1. Continuous streaking method

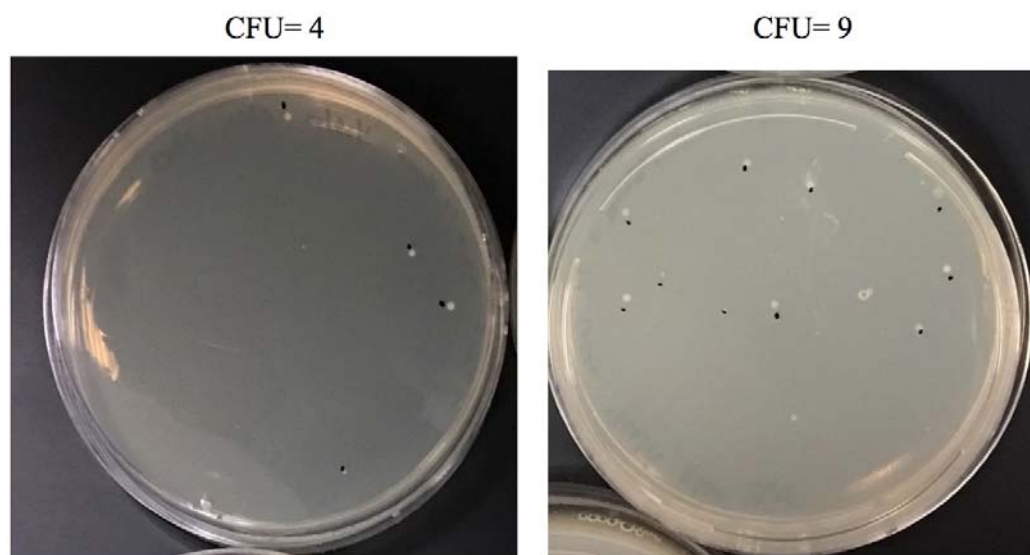


Figure 2. The plates are the example for the quantification of Colony Forming Units (CFU).

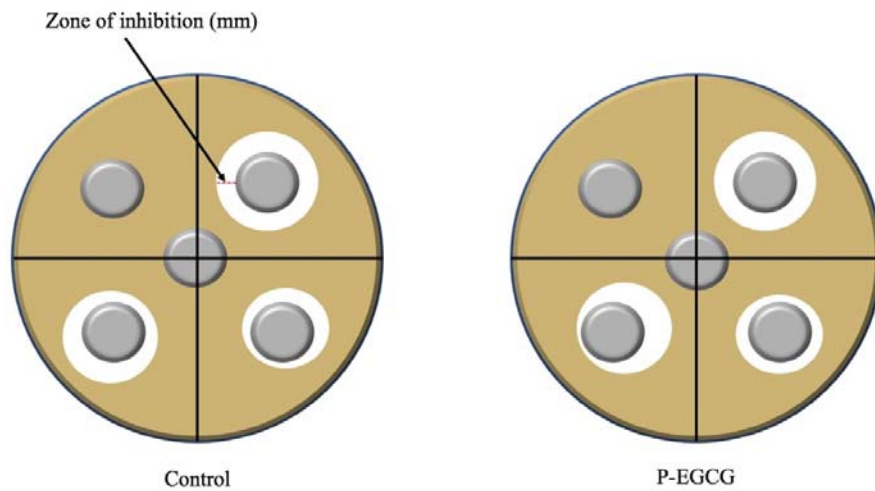


Figure 3. Illustration of a disk diffusion plate. The grey circles represent the disks soaked with different treatments. The white circles represent the clearing zone, which mean no bacteria grow in this section. The red dash line represents the zone of inhibition.

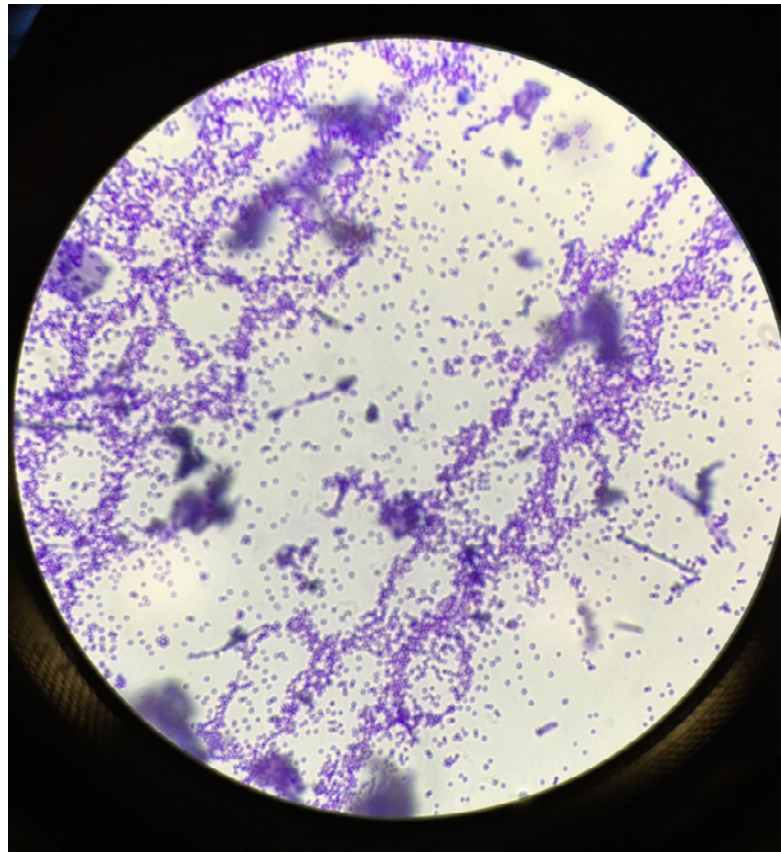


Figure 4. A simple stain of *Streptococcus mutans* viewed under 400x magnification.

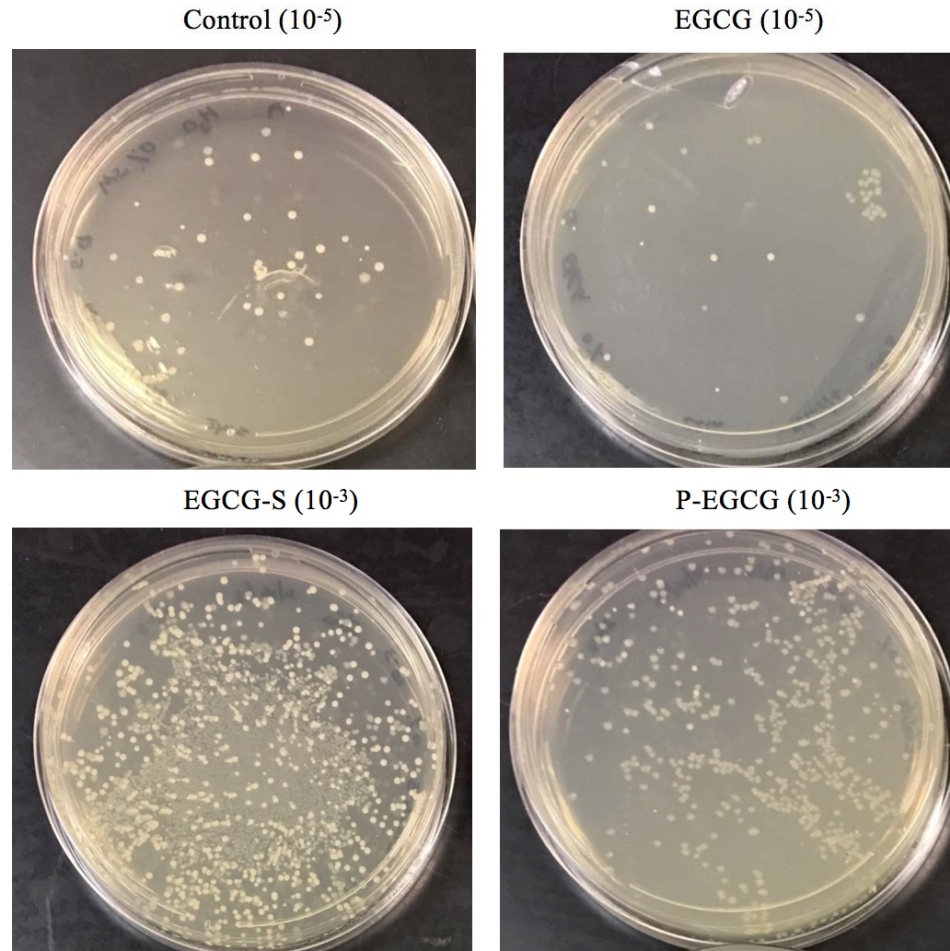


Figure 5. *Streptococcus mutans* with 0% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 2 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

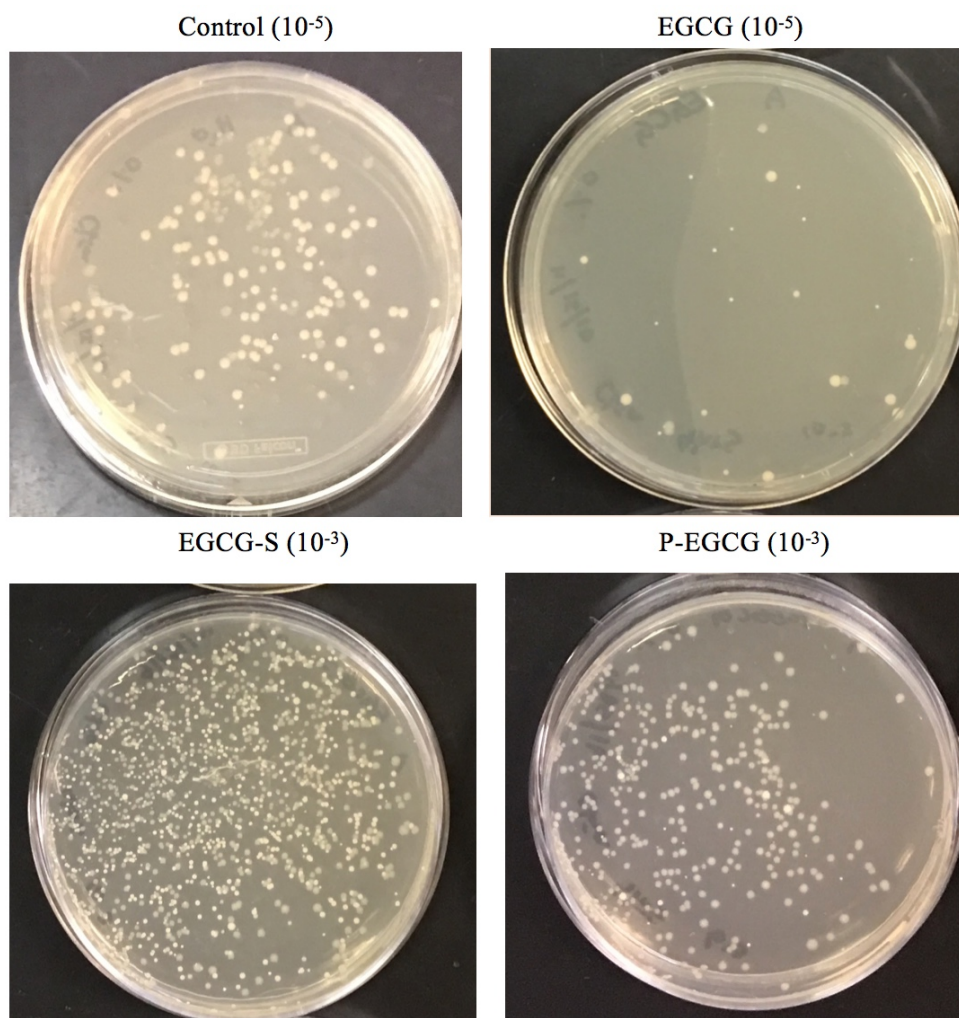


Figure 6. *Streptococcus mutans* with 0% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 4 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

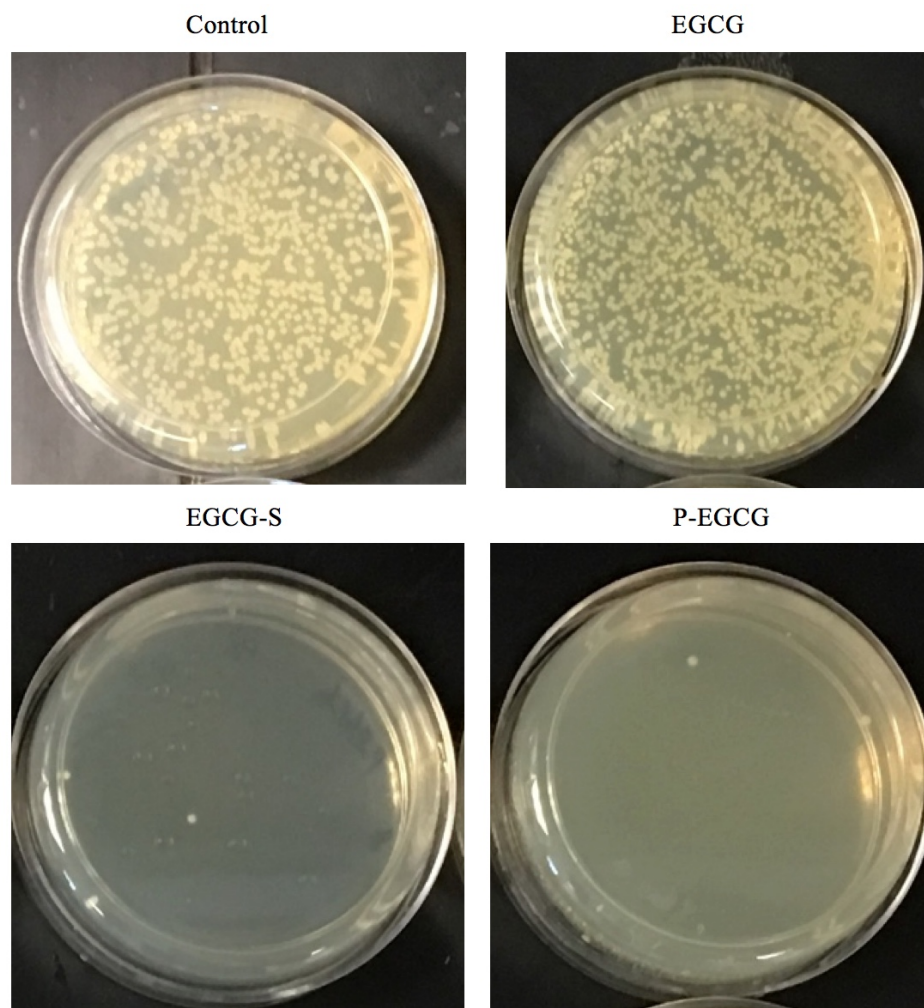


Figure 7. *Streptococcus mutans* with 0% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 24 hours. Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*. Time is also one of key factor for EGCG-S and P-EGCG to inhibit *S. mutans* growth.

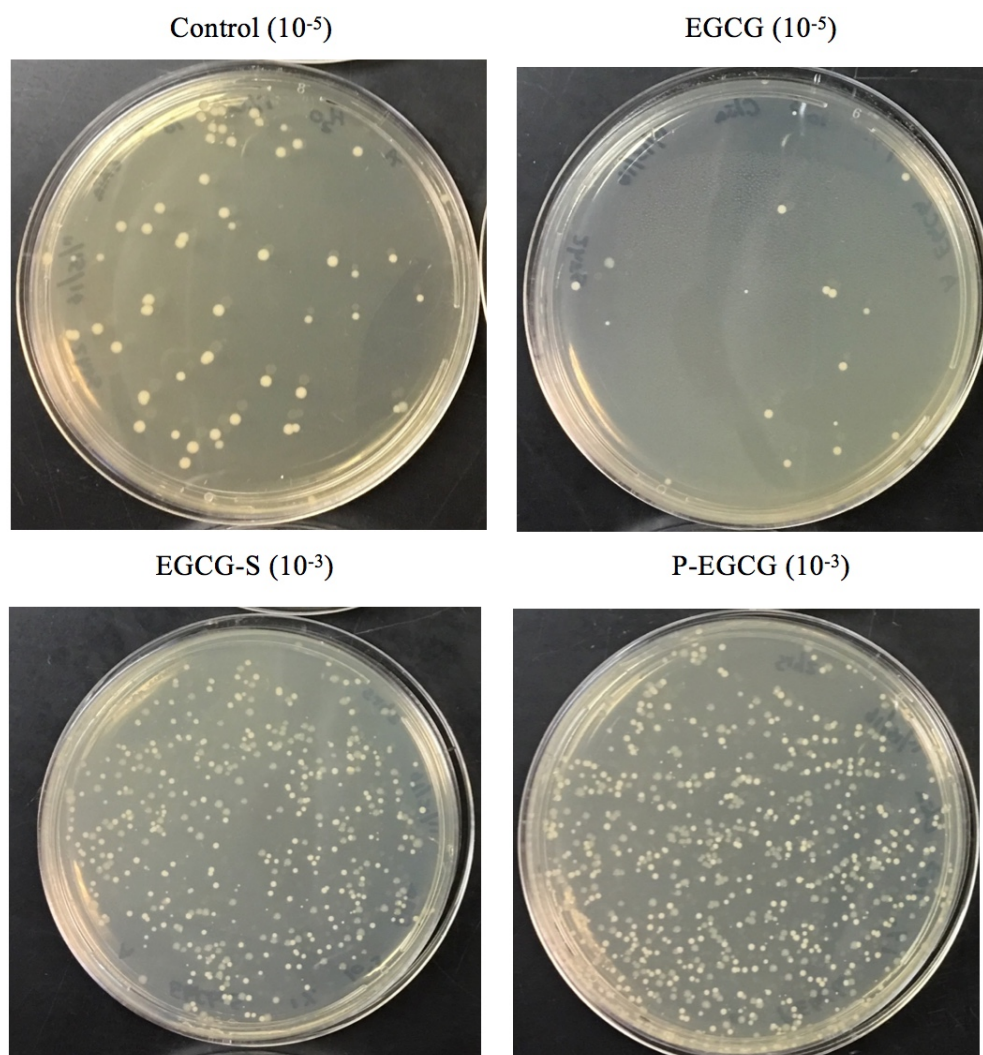


Figure 8. *Streptococcus mutans* with 1% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 2 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

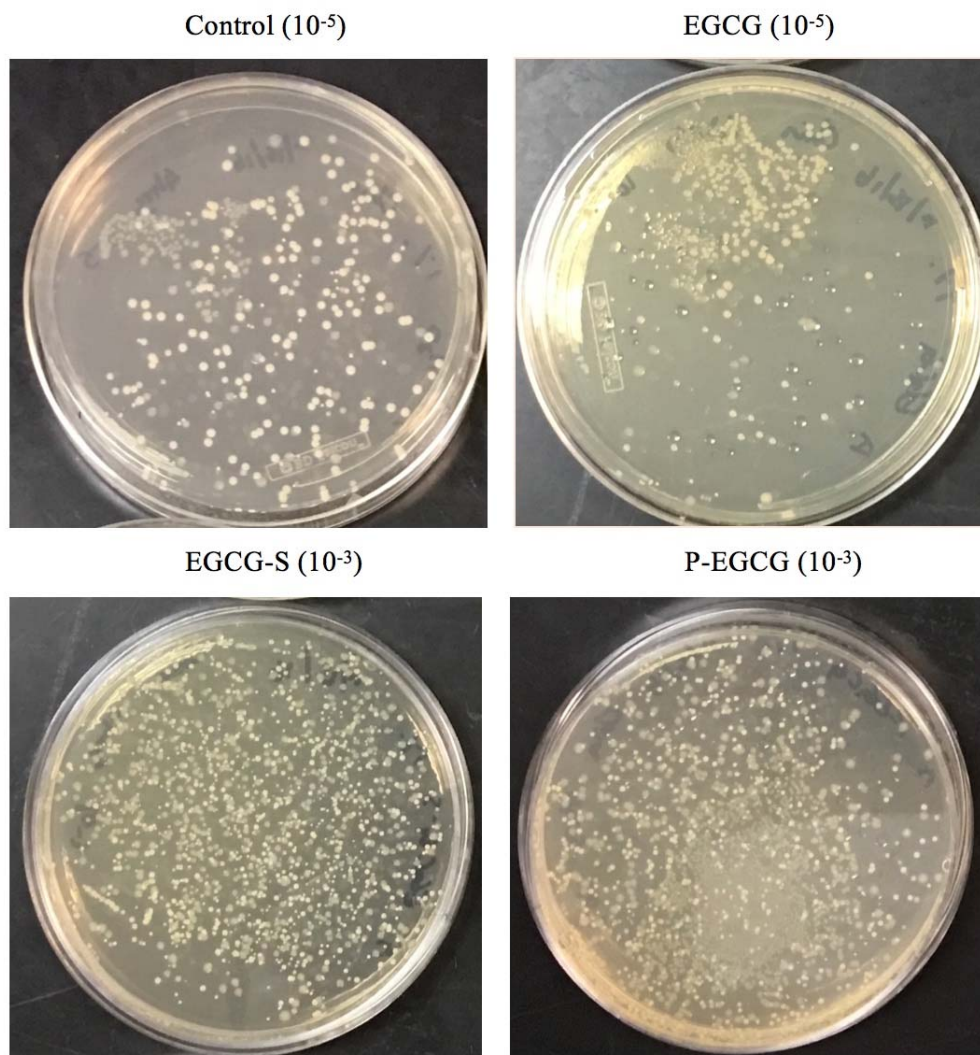


Figure 9. *Streptococcus mutans* with 1% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 4 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

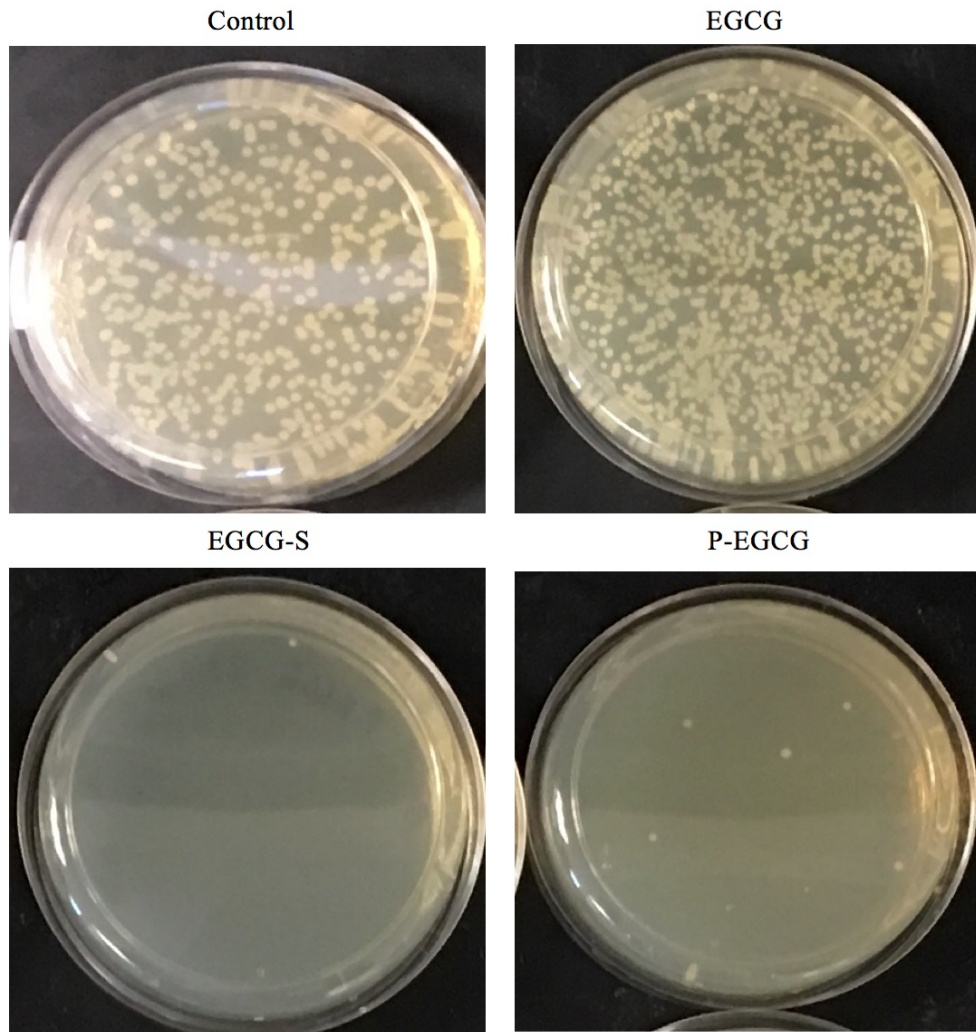


Figure 10. *Streptococcus mutans* with 1% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 24 hours. Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*. Time is also one of key factor for EGCG-S and P-EGCG to inhibit *S. mutans* growth.

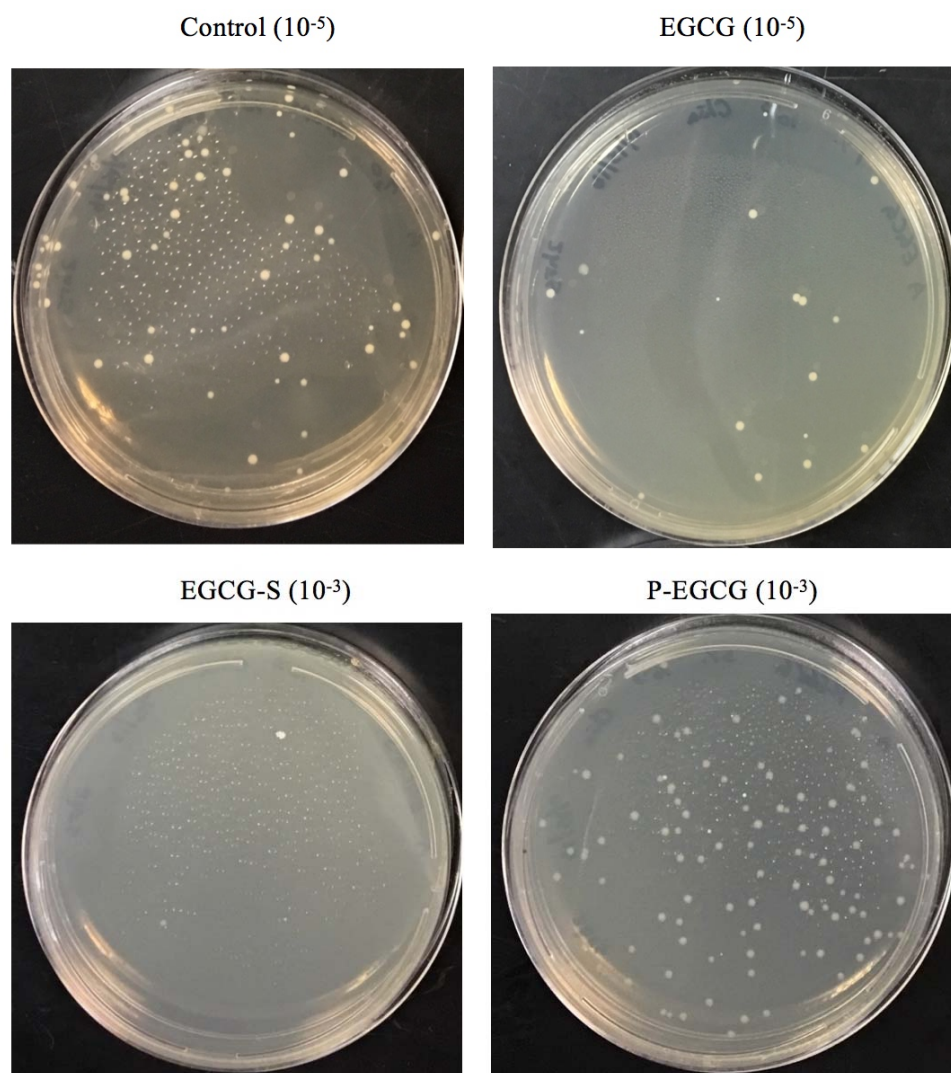


Figure 11. *Streptococcus mutans* with 5% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 2 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

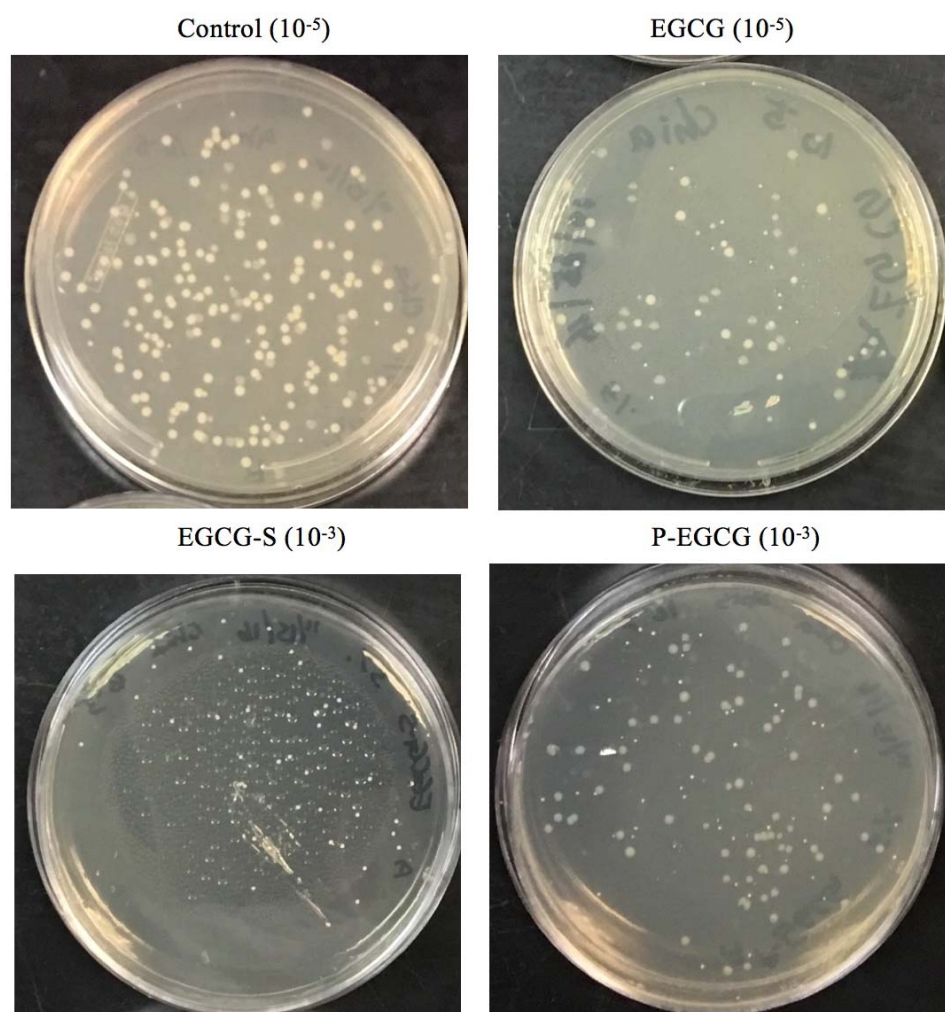


Figure 12. *Streptococcus mutans* with 5% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 4 hours. The serial dilution of the control and EGCG are 10^{-5} . The serial dilution of the EGCG-S and P-EGCG are 10^{-3} . Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

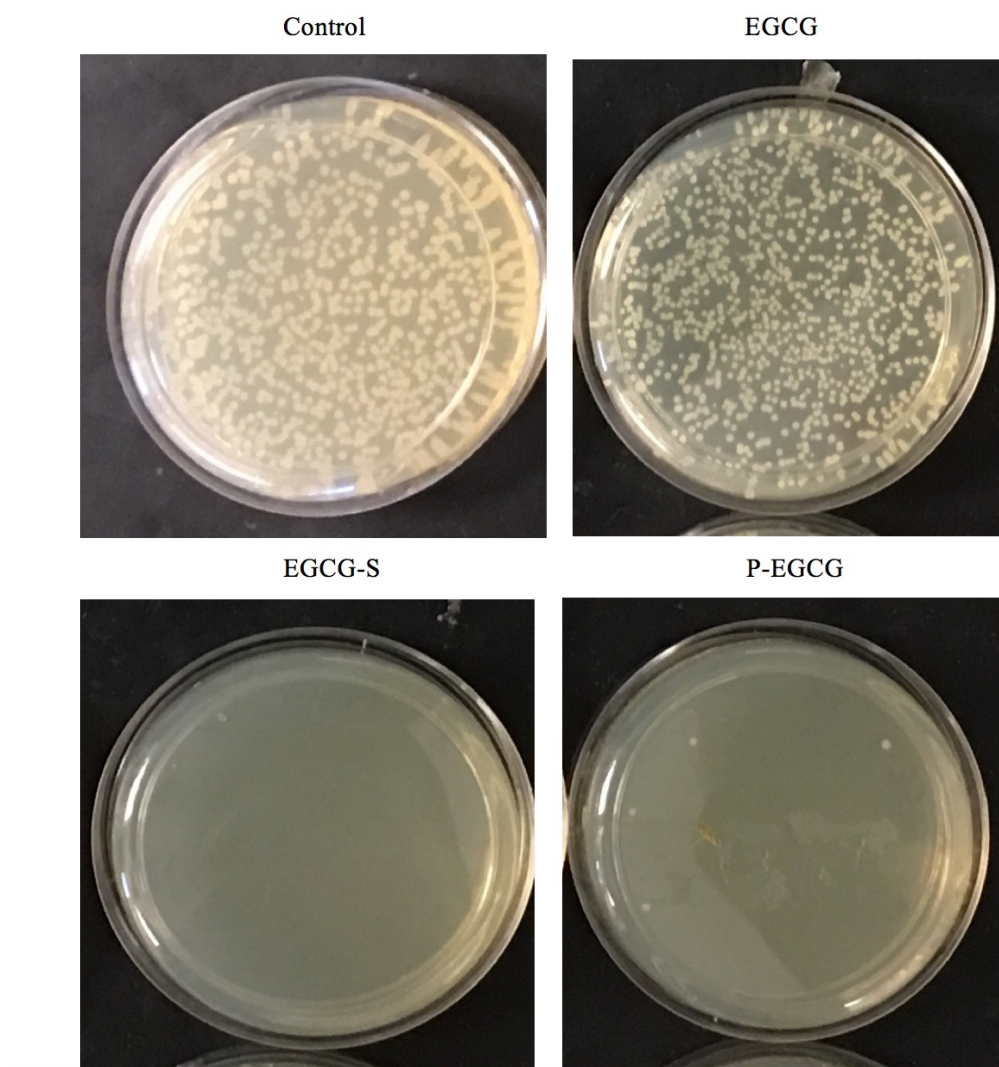
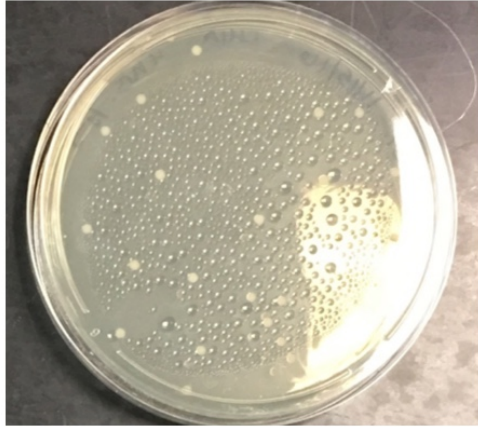
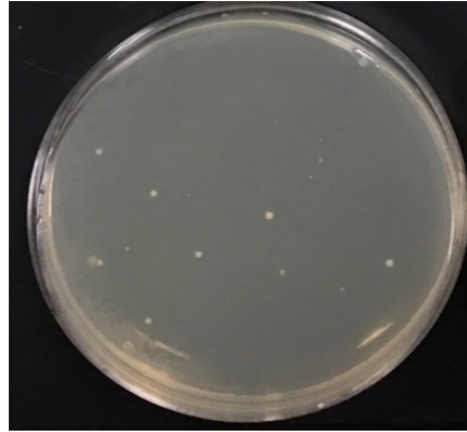


Figure 13. *Streptococcus mutans* with 5% of sucrose was treated with EGCG, EGCG-S, and P-EGCG over 24 hours. Based on the number of colonies formed, demonstrate EGCG-S and P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*. Time is also one of key factor for EGCG-S and P-EGCG to inhibit *S. mutans* growth.

Nutrient Broth



DI H₂O



Cacao liquid

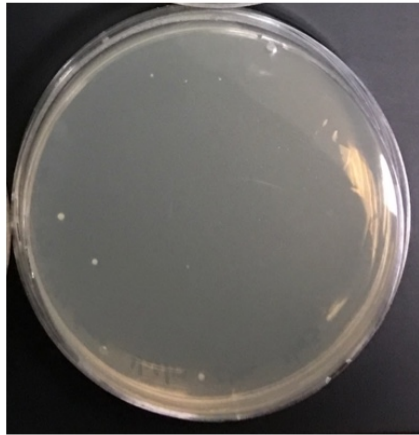
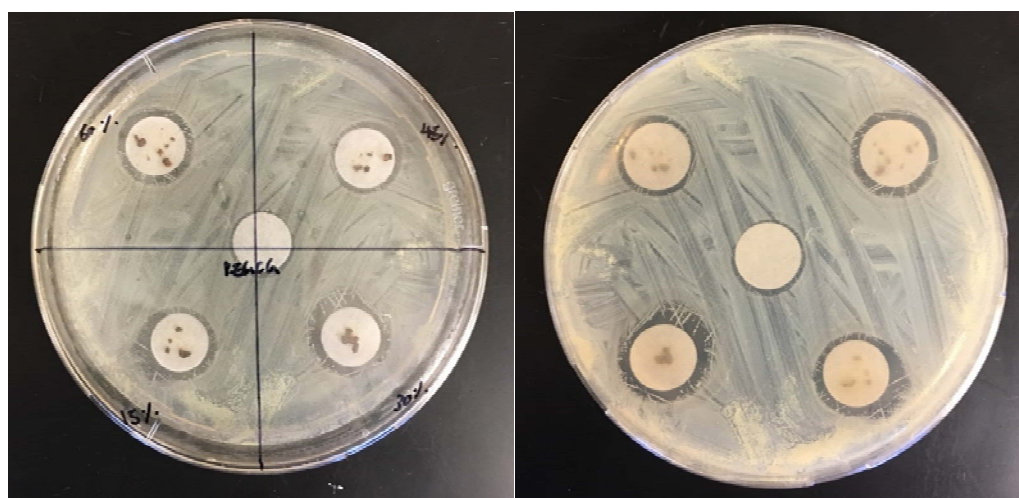


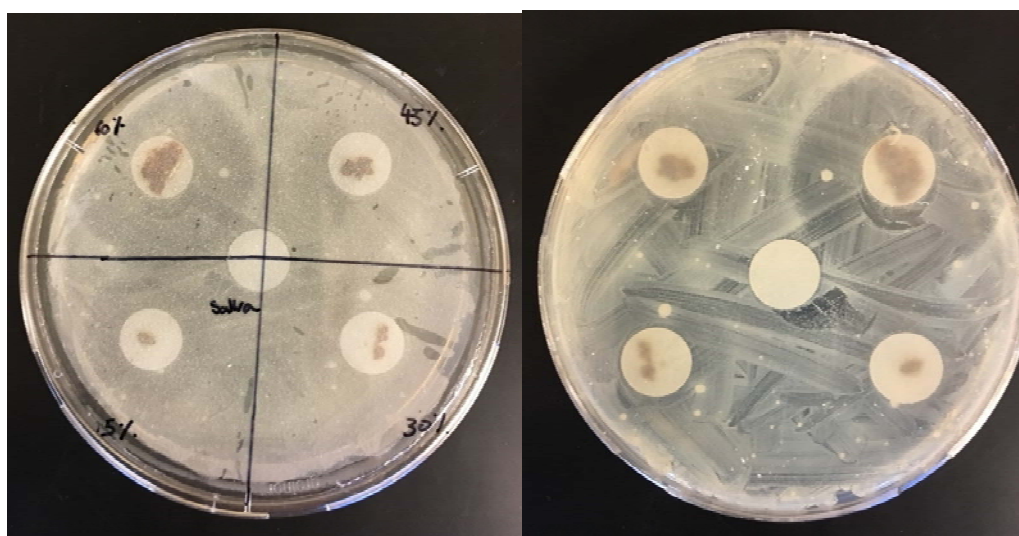
Figure 14. *Streptococcus mutans* growth in nutrient broth, DI H₂O, and cacao liquid. Based on the number of colonies formed, demonstrate cacao liquid has no effect on the growth of *S. mutans*.



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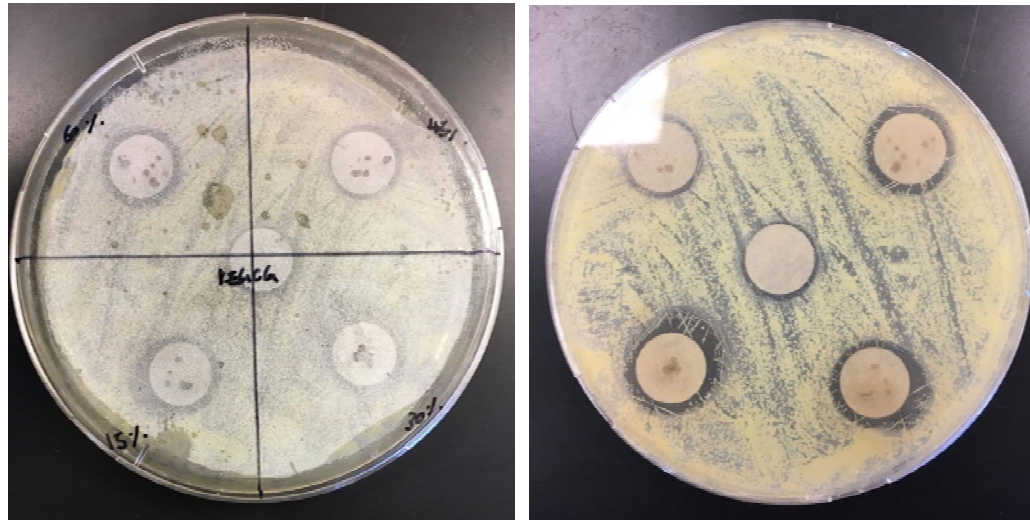
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(b)

Figure 15. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 24 hours of incubation period at 37°C, the zone of inhibition was measured.

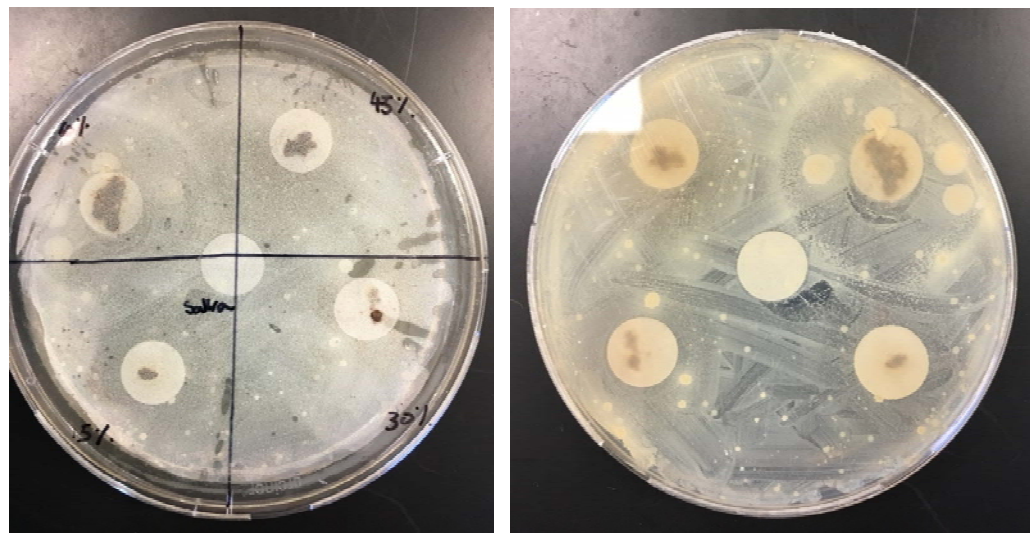
(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.



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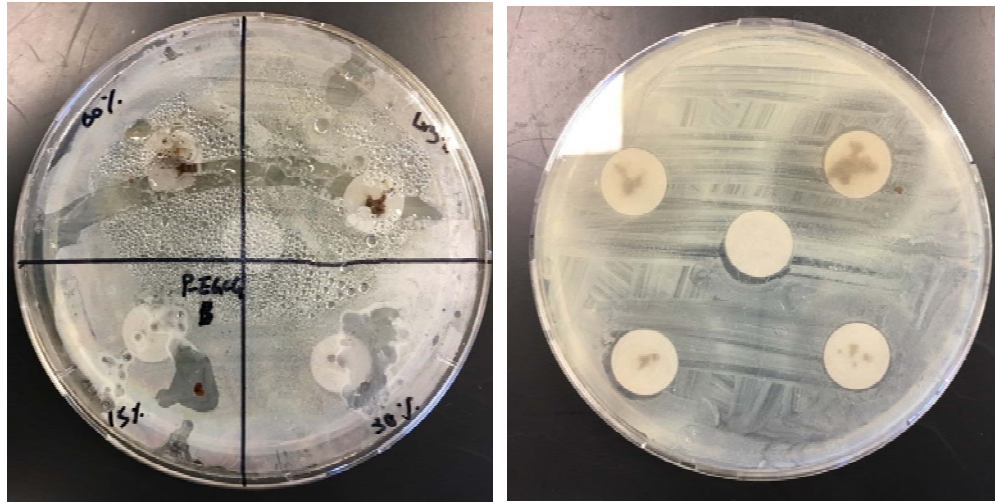
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Figure 16. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 48 hours of incubation period at 37°C, the zone of inhibition was measured.

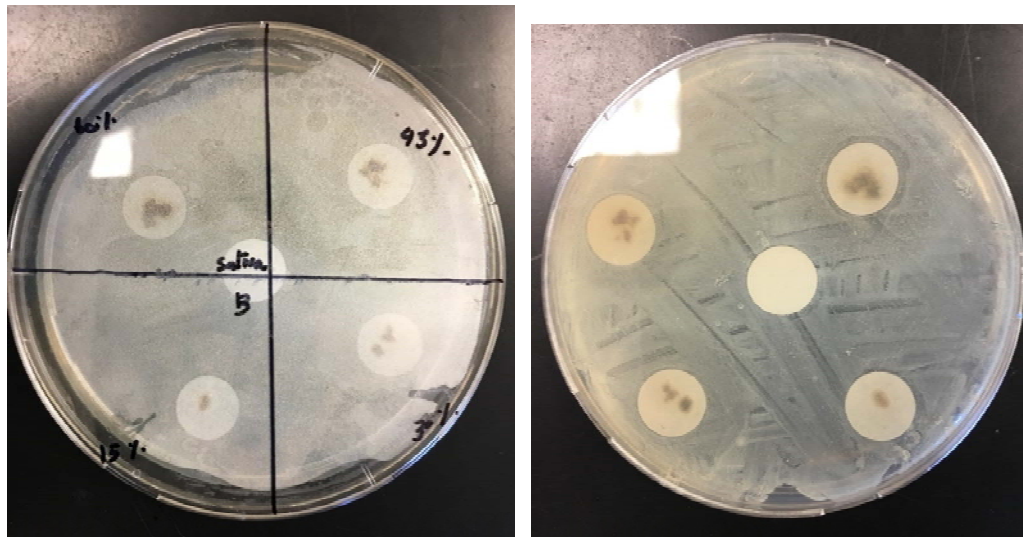
(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.



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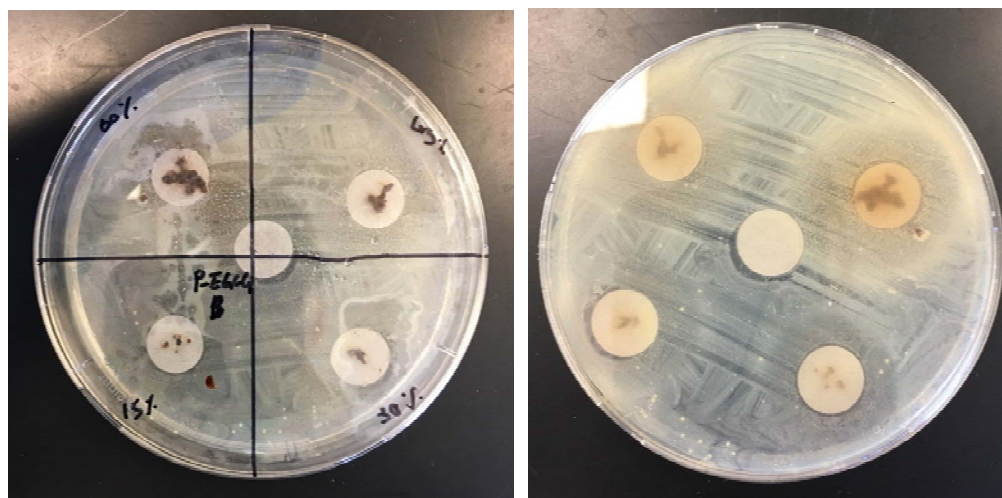
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Figure 17. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 24 hours of incubation period at 37°C, the zone of inhibition was measured.

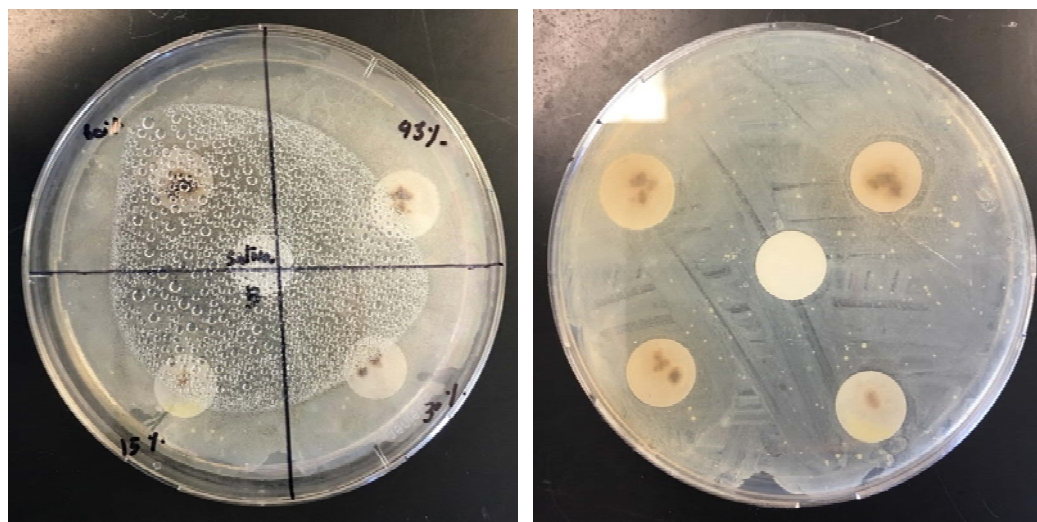
(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.



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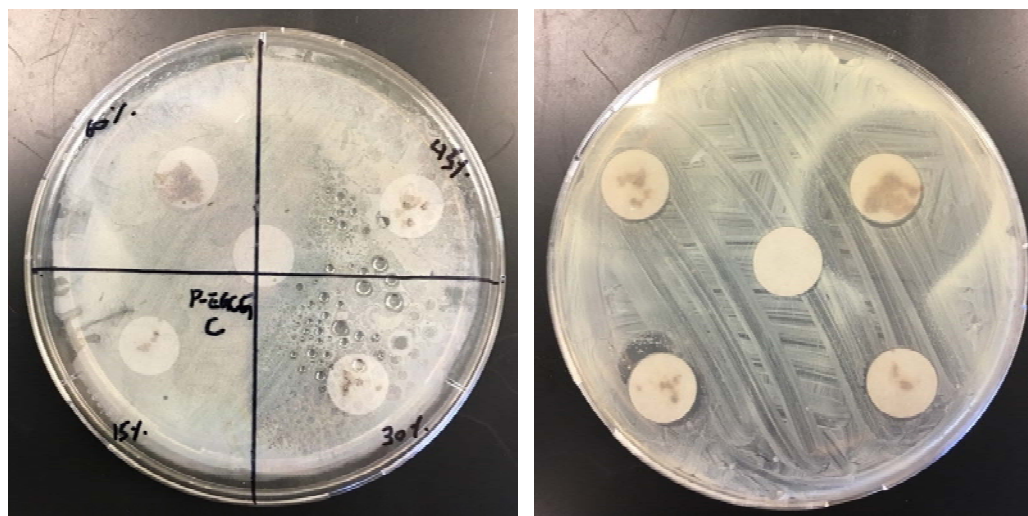
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(b)

Figure 18. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 48 hours of incubation period at 37°C, the zone of inhibition was measured.

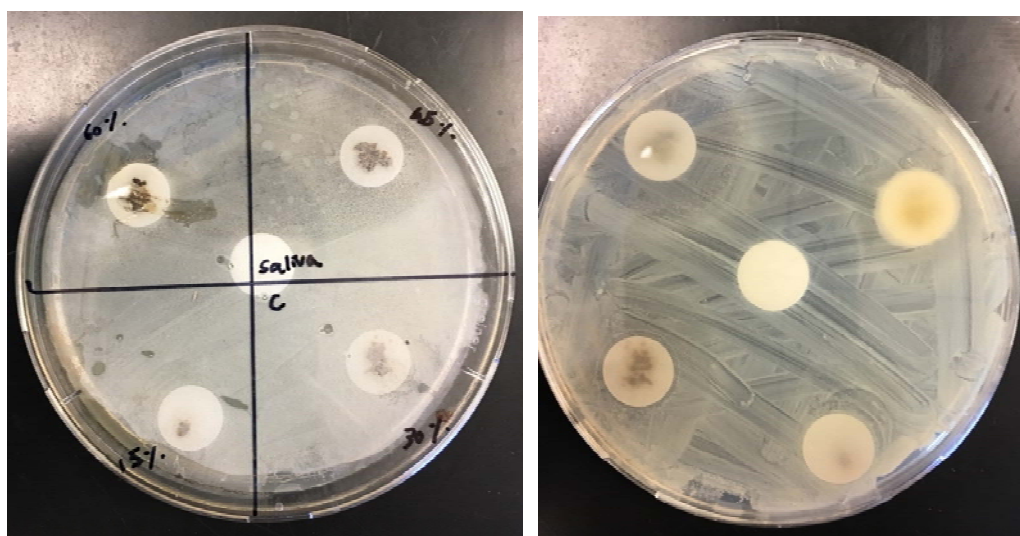
(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.



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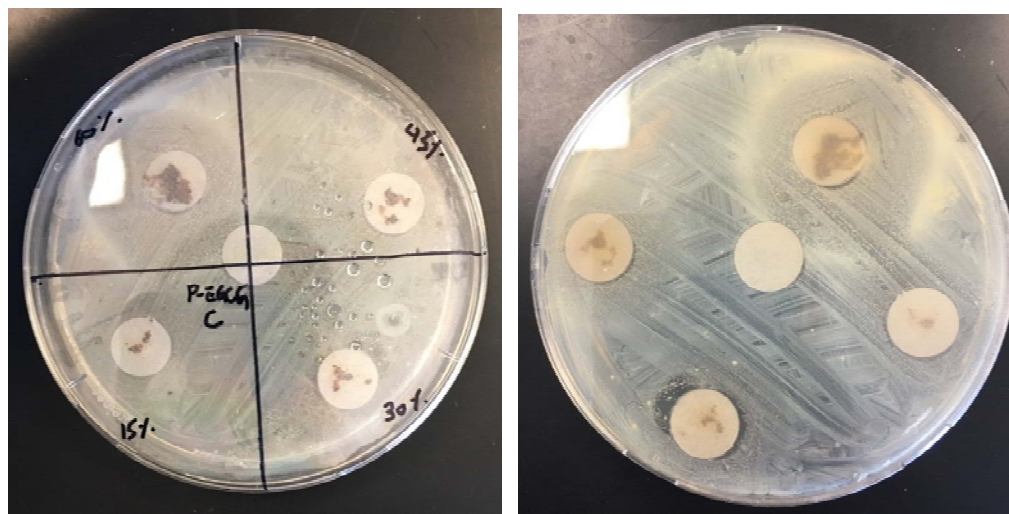
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(b)

Figure 19. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 24 hours of incubation period at 37°C, the zone of inhibition was measured.

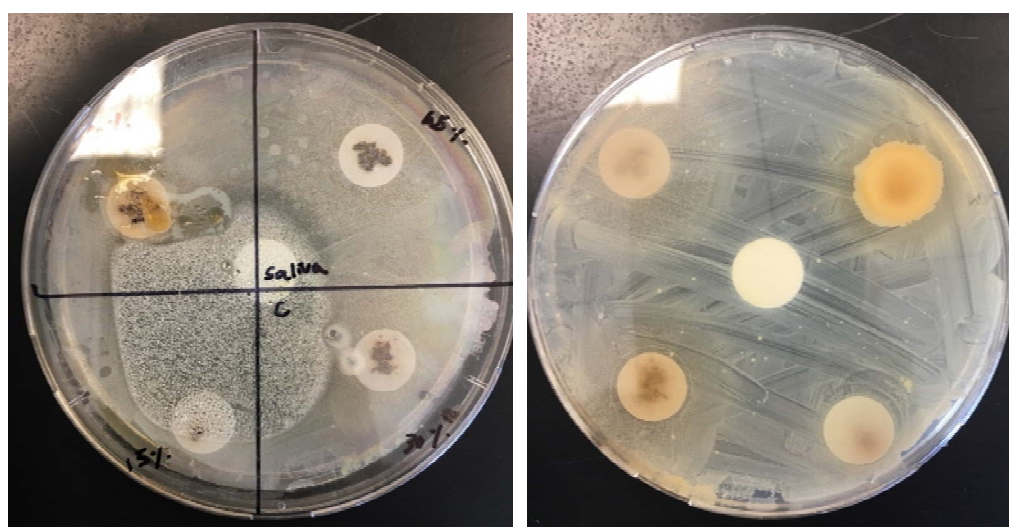
(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.



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Back

(b)

Figure 20. The disk diffusion method was carried out on nutrient agar plates streaked with *Streptococcus mutans*. Disks were added with different concentration of dark chocolate, artificial saliva, and P-EGCG and placed onto the bacteria plate. After a 48 hours of incubation period at 37°C, the zone of inhibition was measured.

(a) shows the disks added with P-EGCG (250ug/mL) is effective by inhibiting the *S. mutans*. (b) shows the ineffectiveness of disks added with DI H₂O.

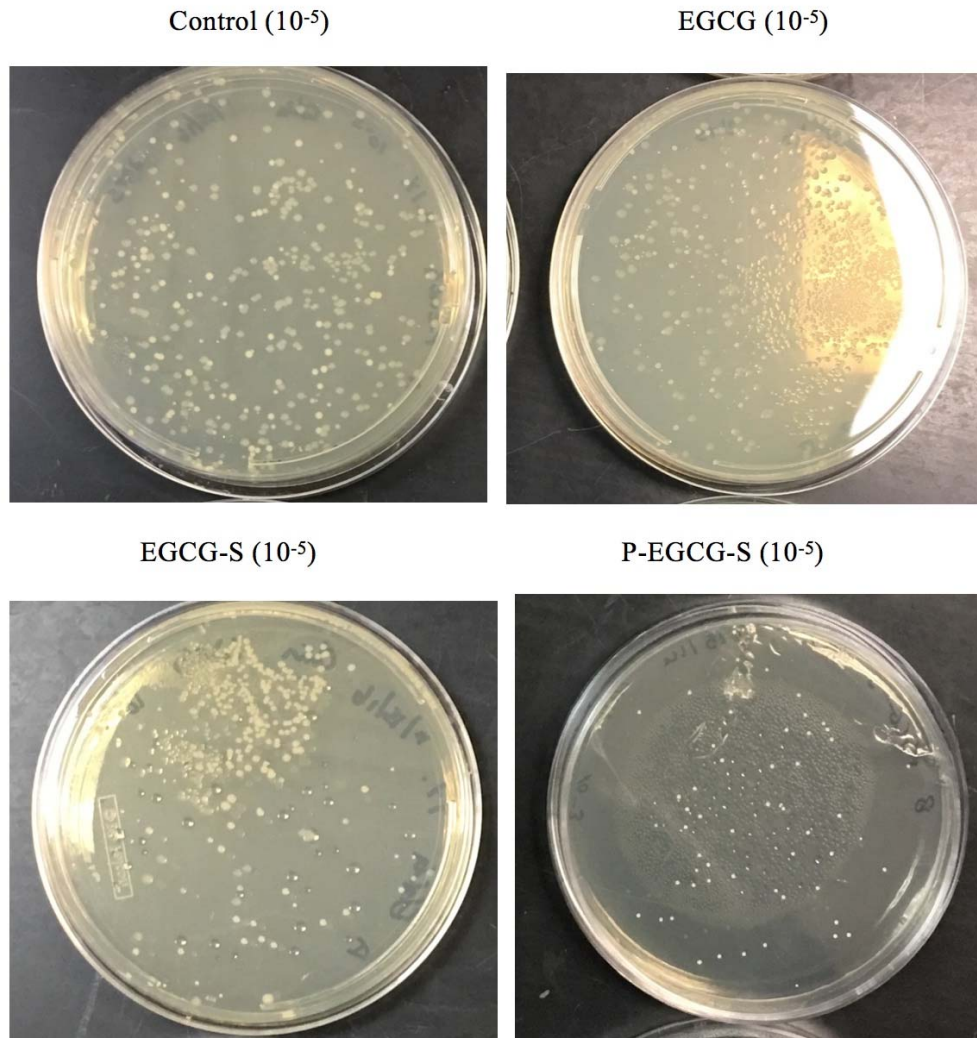


Figure 21. *Streptococcus mutans* with 60% of dark chocolate was treated with EGCG, EGCG-S, and P-EGCG over 2 hours. Based on the number of colonies formed, demonstrate P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.

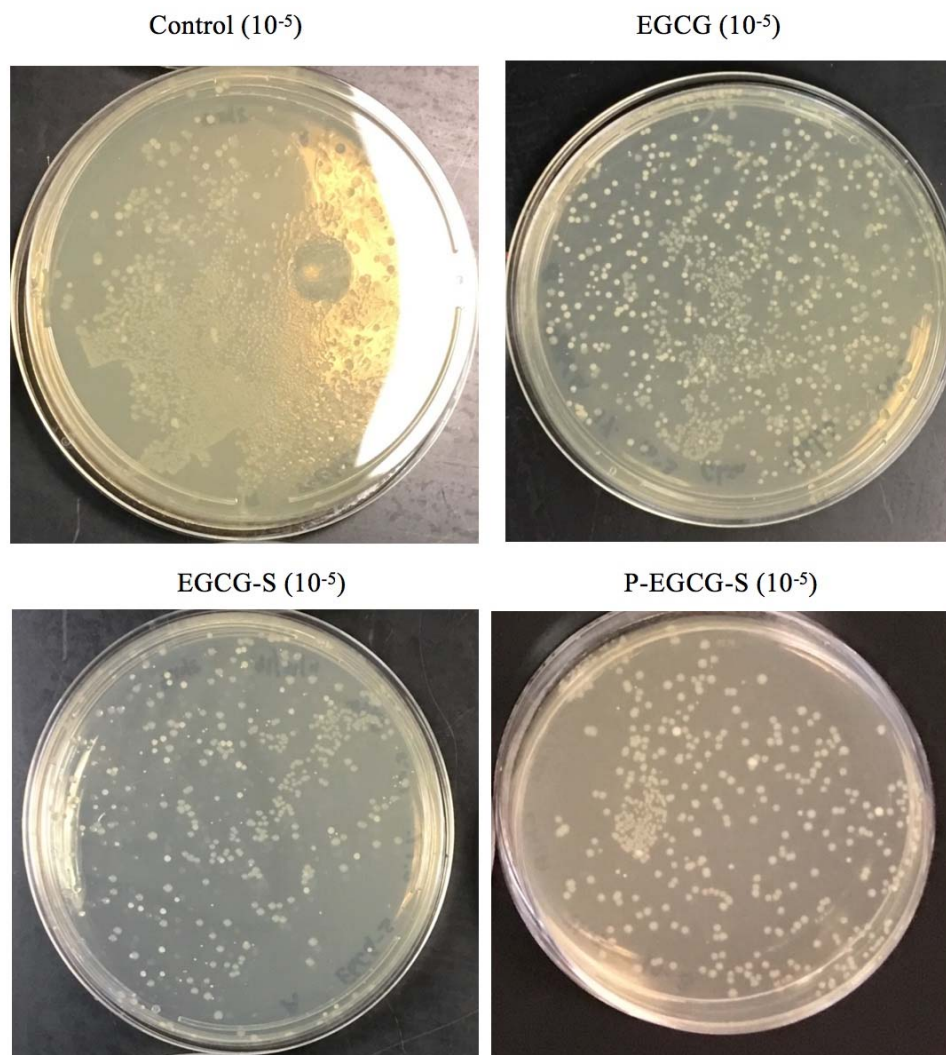


Figure 22. *Streptococcus mutans* with 70% of dark chocolate was treated with EGCG, EGCG-S, and P-EGCG over 2 hours. Based on the number of colonies formed, demonstrate P-EGCG are more effective than EGCG as inhibit the growth of *S. mutans*.